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71 Applicant: **CONNAUGHT LABORATORIES LIMITED**  
**1755 Steeles Avenue West**  
**Willowdale Ontario M2R 3T4(CA)**

72 Inventor: **Klein, Michel Henri**  
**16, Munro Boulevard**  
**Willowdale Ontario M5A 3M2(CA)**  
Inventor: **Boux, Heather Anne**  
**88, Timpson Drive**  
**Aurora Ontario L4G 5N4(CA)**  
Inventor: **Cockle, Stephen Anthony**  
**159, Stephen Street**  
**Richmond Hill Ontario L4C 5P9(CA)**  
Inventor: **Loosmore, Sheena May**  
**70, Crawford Rose Drive**  
**Aurora Ontario L4G 4R4(CA)**  
Inventor: **Zealey, Gavin Ross**  
**49, Bay Hill Drive**  
**Concord Ontario L4K 1G8(CA)**

74 Representative: **Burford, Anthony Frederick et al**  
**W.H. Beck, Greener & Co. 7 Stone Buildings**  
**Lincoln's Inn**  
**London WC2A 3SZ(GB)**

54 Genetic detoxification of pertussis toxin.

57 A new method is described for the preparation of a safe, immunogenic and efficacious vaccine for protection against the disease pertussis. In development of this vaccine, specific functional sites of pertussis toxin have been identified, and using this information, defined mutant holotoxins have been produced by site directed mutagenesis of the toxin gene. A number of these toxin analogues are detoxified, retain an immunodominant S1 epitope, are immunogenic and are protective in the standard pertussis vaccine potency test in mice.

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## GENETIC DETOXIFICATION OF PERTUSSIS TOXIN

FIELD OF INVENTION

5 The present invention relates to a novel method for the detoxification of pertussis toxin by the genetic manipulation of DNA segments coding for one or more amino acid residues essential for the toxin's biological activity. It also relates to a procedure for the creation of genetically altered Bordetella pertussis bacteria that produce the said detoxified pertussis toxin.

BACKGROUND OF THE INVENTION

10 Whooping cough, or pertussis, is a severe, highly contagious respiratory disease of infants and young children caused by infection with Bordetella pertussis. Owing to the many virulence factors associated with this organism, the pathogenesis of the disease is still not fully understood; however, it is generally recognized that the major systemic effects are caused by pertussis toxin (PT). This material exhibits a wide range of biological activities as illustrated by such alternative names as lymphocytosis-promoting factor, histamine-sensitizing factor and islet-activating protein. Many of these effects are associated with its  
 15 biochemical function as an adenosine diphosphate (ADP)-ribosyltransferase. ADP- ribosylation of certain acceptor guanosine triphosphate-binding proteins leads to a loss of control over a variety of metabolic pathways mediated by cyclic adenosine monophosphate and by phospholipase C. In the absence of a protein acceptor, PT also catalyses the hydrolysis of nicotinamide adenine dinucleotide (NAD glycohydrolase activity).

20 Conventional killed whole-cell pertussis vaccines contain a mixture of antigens and there has been a great deal of work towards the development of a defined acellular vaccine comprising specific protective antigens. PT is the most significant protective antigen. Other antigens under consideration are agglutinogens and filamentous hemagglutinin (FHA).

Normally PT and other antigens are chemically inactivated, or toxoided, using agents such as  
 30 formaldehyde, glutaraldehyde or hydrogen peroxide. This approach has the serious disadvantage that a delicate balance must be sought between too much and too little chemical modification. If the treatment is insufficient, the vaccine may retain residual toxicity owing to the presence of a small proportion of unchanged virulence factors including PT. If the treatment is too excessive, the vaccine may lose potency because its native immunogenic determinants are masked or destroyed. This problem is of particular  
 35 concern in the case of PT, since the catalytic subunit is comparatively difficult to inactivate by aldehydes. The possible residual toxicity or reversion of toxoided whole-cell pertussis vaccines has been questioned for many years, and it has suggested that in rare cases the vaccine might cause major neurological damage. All pertussis vaccines that are in use at present, or in the trial stages, depend on the inactivation of the antigens by chemical means, which introduces the problems previously mentioned. It is obvious that if an  
 40 inactivated vaccine could be designed without resorting to the toxoiding process, but preserving the native structure of immunogenic and protective epitopes, an additional degree of safety and efficacy would be added. For these reasons the inventors have genetically manipulated the gene coding for PT, and constructed strains of B pertussis that secrete non-toxic PT analogues.

In its structural organization, PT belongs to the family of ADP-ribosyltransferase bacterial toxins, which  
 45 also includes diphtheria toxin, Pseudomonas aeruginosa exotoxin A, cholera toxin and Escherichia coli heat labile toxin. Accordingly, it consists of two functional moieties: an A portion, which carries the enzymic activity, and a B portion, which binds to the host cell and permits translocation of the A portion to its site of action. In PT, the A portion is a discrete subunit, commonly denoted S1. The B portion is a non-covalent oligomer of five polypeptides arranged as two dimers, comprising subunits S2 plus S4 and subunits S3 plus  
 50 S4 respectively, held together by a joining subunit S5.

The amino acid sequence of the S1 subunit reveals several features of interest. There are only two cysteine residues which form an intrachain disulphide bond; however, it is known that for enzymic activity the toxin must be reduced (Moss et al., J.Biol.Chem. 258, 11872, [1983]), indicating the importance of these residues. There are two tryptophans in S1, and it has been suggested that tryptophan residues are close to the NAD binding sites of diphtheria toxin and P. aeruginosa exotoxin A. Two conserved regions in S1 are

also found in the amino acid sequences of cholera toxin and *E. coli* heat labile toxin (Locht & Keith, Science, 232, 1258, [1986]). In addition the NAD active sites of diphtheria toxin and *P. aeruginosa* exotoxin A have been shown to contain a glutamic acid residue (Carroll & Collier, Proc. Nat. Acad. Sci., U.S.A., 81, 3307, [1984]; Carroll & Collier, J.Biol.Chem., 262, 8707, [1987]).

As noted above, the B portion of PT mediates its binding to cellular receptors and contains two dimers. Whether each of these dimers bears a binding site remains controversial. However, the S2 and S3 subunits are similar in amino acid sequence and binding studies have indicated that lysine and/or tryptosine residues of S3 in particular are implicated in the interaction of the toxin with its receptor. (Nogimori et al., Biochem., 25, 1355, [1986]; Armstrong & Peppler, Infect. Immun., 55, 1294, [1987]).

Site-directed mutagenesis of diphtheria toxin and *P. aeruginosa* exotoxin A at the NAD-interacting glutamic acid residues has led to significant reduction in ADP-ribosyltransferase activity (Tweten et al., J.Biol.Chem., 260, 10392, [1984]; Douglas & Collier, J.Bacteriol., 169, 4987, [1987]). Complete truncated forms of S1 and S2 have been expressed in *E. coli* (Locht et al., Infect. Immun., 55, 2546, [1987]). Mutations of the PT operon generated by transposon insertion, gene truncation or linker insertion have been introduced by allelic exchange into the chromosome of *B. pertussis* (Black et al., Ann. Sci. 175, [1986]; Black & Falkow, Infect. Immun., 55, 2465, [1987]). However, the biological and immunoprotective properties of fully-assembled recombinant holotoxins specifically detoxified by site-directed mutagenesis of functional amino acid residues have not been reported. The generation of such PT analogues for inclusion in a safe and efficacious pertussis vaccine is the subject of this invention

In testing for the efficacy and toxicity of materials that could be candidates for a protective vaccine, there are a number of *in vivo* and *in vitro* assays available. The standard test for potency is the mouse protection test, which involves intra-cerebral challenge with live *B. pertussis*. Newer vaccine tests measure the production of protective antibodies. A common toxicity test is the CHO (Chinese hamster ovary) cell clustering assay, which reflects both the ADP-ribosyltransferase and binding ability of the toxin (Burn et al., Infect. Immun., 55, 24, [1987]). A direct test of the enzymic activity of PT is the ADP-ribosylation of bovine transducin (Walkins et al., J. Biol. Chem., 260, 13478, [1985]).

### SUMMARY OF INVENTION

In accordance with the present invention, there is provided a novel method of detoxifying PT, which does not suffer from the drawbacks of the prior art chemical methods and yet provides an detoxified PT that retains its immunological properties without possessing undesirable side effects. In the present invention, amino acid residues of the toxin that are crucially important to its functional and toxic activities are identified. These residues are subsequently removed or replaced by site-directed mutagenesis of the isolated toxin gene. The mutated toxin operon resulting from such manipulations then is substituted for the native gene in the organism, which thereby produces the decreased toxicity toxin under normal growth conditions. In this manner, the three-dimensional structure and thus the immunogenicity of the PT analogue is minimally impaired. Indeed, an appropriate mutant form of the toxin on its own may provide satisfactory protection against the severe symptoms of pertussis, though other components may be required to establish resistance against the bacterial infection itself.

In accordance with one aspect of the present invention, therefore, there is provided an immunoprotective genetically-detoxified mutant of pertussis toxin. By the term "genetically-detoxified" as used herein is meant a pertussis toxin mutant which exhibits a residual toxicity of about 1% or less, preferably less than about 0.5%, of that of the native toxin. The residual toxicity is determined by CHO cell clustering assay and ADP-ribosyltransferase activity.

In accordance with another aspect of the present invention, there is provided a vaccine against *Bordetella pertussis* comprising an immunogenically-effective amount of the immunoprotective mutant of pertussis toxin or a toxoid thereof and a physiologically-acceptable carrier therefor. The genetically-detoxified pertussis toxin also may be used as a carrier protein for hapten, polysaccharides or peptides to make a conjugate vaccine against antigenic determinants unrelated to the toxin.

A further aspect of the present invention provides a method of production of the mutant, which comprises identifying at least one amino acid residue of the toxin which confers toxicity to the toxin; effecting site-directed mutagenesis of the toxin gene to remove or replace at least one such residue and to produce a mutated toxin operon; substituting the mutated toxin operon for the native gene in the *Bordetella pertussis* organism; and growing the transformed organism to produce an immunoprotective, genetically-detoxified toxin.

As will be apparent from the following disclosure, the present invention further provides novel strains of Bordetella pertussis from which the toxin operon has been removed or has been replaced by a mutant gene as provided herein.

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### BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows the sequence of amino acids obtained by automated sequencing or radiolabelled peptides A and B and are compared with residues from mature S1;

Figure 2 shows the structures of various TOX clones obtained from the chromosomal libraries;

Figure 3 shows the construction of subclones containing the TOX gene from the genomic clone  $\lambda$  gtl 15-4-1, with the TOX gene being inserted into the multiple cloning site of pUC8:2, which contains Bgl II and Xba I sites;

Figure 4 shows the construction of subclones of the TOX gene used for sequencing the operon. In (a), a restriction map of the TOX gene and the protein subunits are indicated, with clones being derived from the pUC8:2/TOX clone J-169-1, and the subunit genes being subcloned into M13mp18, M13mp19 or pUC8:2, as indicated; in (b), clones of the 5' region of pUC8:2, S1 in M13mp18 and S1 in M13mp19 clones are described; in (c), clones of S2 in M13mp18 and M13mp19 is shown; in (d), clones of S4/S5 in M13mp18 and M13mp19 are shown; and, in (e), clones of S3 and the 3' region in M13mp18 and pUC8:2;

Figure 5 shows the nucleotide sequence and structural gene translation products of the B. pertussis 10536 TOX gene;

Figure 6 shows the construction of TOX or TOX analogue genes in the broad-host-range plasmid pRK404 (Ditta et al., Plasmid, 13, 149, [1985]). In (a) and (b), there is shown the construction of primary TOX analogue genes in pRK404 from mutated genes and native genes, while in (c), there is shown a typical construction of a "crossed" mutant from two S1-mutated genes;

Figure 7 shows the development of a "suicide" plasmid, one capable of conjugative transfer but not replication, based on pRK404 and pMK204 (Kahn et al., Methods in Enzymology, 68, 278, [1979]), for non-homologous recombination, the final plasmids also containing a Tn5-derived kanamycin resistance gene 3' to the TOX or TOX analogue genes;

Figure 8 shows the cloning of the 5' and 3' flanking region of the TOX gene. (a) shows the construction of the 5' portion of TOX in pUC8:2 from the  $\lambda$  Charon 35 clone Ch421; (b) shows the construction of the 3' portion of TOX in pUC8:2 from  $\lambda$  Ch 111; and (c) shows the generation of a pUC8:2 clone containing TOX plus its 5' and 3' flanking regions;

Figure 9 shows the construction of plasmids for the deletion of the TOX operon from the B. pertussis chromosome by homologous recombination; and

Figure 10 shows the construction of plasmids for reintegration of TOX analogues into the B. pertussis genome by homologous recombination, the final plasmids being based on the suicide plasmid shown in Figure 7 and containing the tetracycline resistance gene from pRK404 placed 3' to the TOX analogue gene.

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### GENERAL DESCRIPTION OF INVENTION

It has been shown that the TOX operons from different strains of B. pertussis are nearly identical in sequence (Nicosia et al, Proc. Nat. Acad. Sci., U.S.A., 83, 4631, [1986]; Loch & Keith, Science, 232 1258, [1986]). The TOX locus is here defined as a DNA fragment beginning at the EcoR I cleavage site which encodes a 5' flanking sequence, the promoter region, the structural genes for all PT subunits and a 3' flanking sequence. The TOX gene from B. pertussis 10536, which is the strain used by the inventors, was cloned and sequenced. Its nucleic acid sequence was found to be highly homologous to other published sequences, with four unique base differences downstream from the G of the EcoR I site defined as base 1. The complete nucleotide and corresponding amino acid sequence of the structural genes are shown in Figure 5.

The plasmid DNA of clone J-169-1 which contains the TOX gene from Bordetella pertussis 10536 cloned into pUC8:2 as a 4.6 kb EcoR I, BamHI fragment, has been deposited with the American Type Culture Collection (ATCC) in Rockville, Maryland, U.S.A. on November 23, 1988 under American Number 40518.

The T at position 315 is unique to strain 10536 and there are three differences in the S1 gene at

positions 710, 1200 and 1202, resulting in two unique amino acids, glutamic acid and valine, at positions 34 and 198 of the mature S1 sequence, respectively. The toxin genes of *B. paraptussis* and *B. bronchiseptica* are not expressed because of multiple mutations in their promoter regions, (Arico & Rappuoli, J.Bacteriol., 169, 2849, [1987]). This has allowed the use of *B. paraptussis* as a host for the expression of mutated toxin genes for screening purposes.

The inventors have shown that substitution of a single amino acid in S1, in particular at the active site for NAD hydrolysis (position 129), virtually abolishes the ADP-ribosyltransferase activity of PT. However, it may be desirable to alter several sites on the holotoxin to ensure complete safety. Accordingly, this invention applied to single or multiple mutations in both or either of the A and B portions of the toxin to abolish toxicity, and to the reinsertion of these mutations back into the genome of *Tox<sup>-</sup>* strains of *Bordetella*.

A number of strategies have been used by the inventors to determine regions of the toxin that might be closely associated with its biological activities, and, therefore, contain candidate sites for genetic manipulation.

PT was prepared from culture supernatants of *B. pertussis* (strain 10536). The crude solution was concentrated by ultrafiltration and passed through a fetuin-agarose affinity column to adsorb PT. PT was eluted from the washed column using potassium thiocyanate and dialyzed into a phosphate-saline medium. At this stage, the purity was 90-95%, as determined by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The major contamination was FHA. Further purification was achieved by chromatography through a hydroxyapatite column, giving a material with a purity >99%.

The site of interaction of the S1 subunit with NAD was determined by photo-crosslinking NAD to isolated and purified S1 using [<sup>14</sup>C]NAD, labelled either in the nicotinamide carbonyl group or the adenine moiety. Radiolabel was efficiently absorbed from the nicotinamide moiety into the protein. The protein was then digested with trypsin and chromatographed on an HPLC column, giving two major radioactive peptides. After purification the two tryptic peptides were sequenced and the first fifteen residues corresponded to residues 118-132 of mature S1. In both the peptides, radioactivity was associated with an unidentified amino acid corresponding to position 129 in mature S1. Radioactivity was not detected in any other position. This established that GLU<sup>129</sup> is the site of photo-crosslinking of NAD and is therefore likely to be an important component of the nicotinamide interaction site. Significantly the sites of linkage in diphtheria toxin and *P. aeruginosa* exotoxin A are also glutamic acid residues and the three amino acid sequence commencing at GLU<sup>129</sup> of S1 strongly resembles the analogous sequences of the other bacterial toxins.

Chromosomal DNA was prepared from *B. pertussis* (strain 10536) and was digested with the restriction enzyme EcoR I in such a way that fragments were obtained ranging in size from a few hundred bases to a few kilobases. The DNA fragments were ligated with  $\lambda$  gtlI DNA which had been digested with EcoR I and dephosphorylated. The DNA was packaged into phage particles and maintained in *E. coli* Y1090 as a gtlI *B. pertussis* genomic library. Alternatively, *B. pertussis* chromosomal DNA was digested with the restriction enzyme Sau3A I to generate very large DNA fragments which were ligated with BamH I restricted  $\lambda$  Charon 35 DNA. The DNA was packaged into phage particles and maintained in *e. coli* LE392 as a  $\lambda$  Ch 35 *B. pertussis* genomic library.

The genomic libraries were plated and phage plaques transferred onto nitrocellulose filters. The filters were screened by DNA hybridization using an oligonucleotide probe specific for the PT S4 subunit. Positive plaques were further purified by two additional rounds of plating and hybridization. Phage DNA was prepared from the positive plaques and subjected to restriction enzyme digestion and Southern blot analysis. Clones containing the entire 4.6 kb EcoR I *pertussis* toxin operon (TOX) or portions thereof and with differing 5' or 3'-flanking regions were characterized. The TOX gene was subcloned for sequence analysis and further genetic manipulation. Sequencing was performed using the dideoxy chain termination method and the results indicated four novel bases in the 10536 TOX gene as compared to publishing sequences.

Subclones of S1 or S3 genes in M13 phage were subjected to *in vitro* site-direction mutagenesis using the phosphorothioate procedure. Single-stranded DNA from these clones was annealed with oligonucleotide primers specifically designed to mutate or delete one or more amino acids. The mutagenesis was carried out using a kit available from a commercial source. Mutations were verified by sequencing of single-stranded phage DNA. Mutant subunit genes were recombined with the remainder of the operon to construct mutant holotoxin genes in the broad-host-range plasmid pRK404 maintained in *E. coli* JM109.

In order to characterize the holotoxin analogues, these plasmids were transferred to a spontaneous streptomycin-resistant *B. paraptussis* strain by conjugation on a solid surface, using pRK2013 as a helper plasmid. The colonies were selected on tetracycline-containing Bordet-Gengou blood plates. Mutated genes

were also integrated into the chromosome of *B. parapertussis* by conjugative transfer of a suicide plasmid. The integration was either random or directed through homologous recombination utilizing the flanking regions of the *B. pertussis* TOX operon. Figure 7 shows the construction of a suicide plasmid containing mutants for random recombination.

Liquid cultures were grown in modified Stainer-Scholte medium containing methyl- $\beta$ -cyclodextrin in shake flasks (10 ml to 2L) or in fermentors (20L to 50L). The expression level of holotoxin analogues in culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA) and found to vary with the mutation. The residual toxicity of the analogues was measured by the CHO cell clustering assay.

A number of PT analogues were purified from 2L to 50L cultures of recombinant *B. pertussis* strains, according to methods described in detail for native PT. The ADP-ribosyltransferase activity of these mutants was determined as the extent of incorporation of radioactivity into bovine transducin from [ $^{32}$ P]-labelled NAD. Table 1a below lists the PT mutants generated and Table 1b below summarizes their residual toxicity and enzymic activity.

Selected purified mutants were tested in mice for acute toxicity, histamine sensitization activity and potency in the standard mouse intracerebral challenge test. These results are presented in Table 2 below and show that PT analogues have a markedly-decreased acute toxicity and histamine sensitization activity and that they are immunoprotective in the mouse potency test.

The immunological properties of PT analogues were further investigated by epitope mapping and by analysis of the antibody response in mice. Several monoclonal antibodies (MAbs) specific for individual subunits or dimers of PT were prepared and used to determine by ELISA whether the epitopes defined by these antibodies were affected by the mutations. The S1 epitope recognized by MAb PS21 is of particular significance, since it is immunodominant in mice and this antibody confers passive protection in the mouse intracerebral challenge test. The preservation of this epitope in the PT analogues is indicated in Table 1b.

Immunogenicity studies in mice were performed on three purified PT mutants. Immune sera were tested for their ability to inhibit PT-induced CHO cell clustering (Table 3 below), and for their anti-PT, anti-S1 and anti-B-oligomer antibody titres by indirect ELISA (Table 4 below).

To generate a *B. pertussis* strain expressing a mutated TOX gene suitable for vaccine production, the endogenous TOX operon was deleted by homologous recombination using electroporation of linear *B. pertussis* DNA containing the 5' and 3'-flanking regions of the TOX locus. Selected mutant genes were then reintegrated into the TOX locus of the *B. pertussis* chromosome. Clones containing mutated TOX genes were grown and the culture supernatants assayed for level of expression of PT analogues and their residual toxicity as previously described. These results are shown in Table 5 below.

Certain *Bordetella pertussis* strains wherein the TOX gene has been removed entirely or has been replaced by certain clones, have been deposited with ATCC on November 23, 1988, as follows:

Strain	Modification	ATCC Accession Number
<i>B. pertussis</i> 29-9	TOX deleted (Tox <sup>-</sup> )	53838
<i>B. pertussis</i> S-2962-1-2	S1:GLY129	53837
<i>B. pertussis</i> S-3036-2	S1:GLU58	53835
<i>B. pertussis</i> S-3122-3-1	SA:ALA41	53834
<i>B. pertussis</i> S-3122-2-3	S1:GLY129, S3:ASN92ARG93	53833

The Tox<sup>-</sup> strain is a novel strain of *Bordetella pertussis* from which the toxin operon has been removed and from which foreign DNA is absent and which is capable of being grown in the absence of antibiotics to

produce B. pertussis antigens free of pertussis toxin.

Each of the transformed strains is a strain of Bordetella pertussis in which the toxin operon has been replaced by a mutant gene formed by site-directed mutagenesis of at least one specific amino acid residue responsible for pertussis toxin toxicity.

5 The data presented herein demonstrate that the inventors have produced a series of pertussis toxin analogues that exhibit a substantial reduction in CHO cell clustering and enzymic activities (0.1 to 1% of the wild-type activity). Many of these analogues also maintain an immunodominant S1 epitope recognized by a protective monoclonal antibody. Moreover, certain of these mutants have been shown to protect mice at doses that exhibit minimal toxicity against challenge with virulent B. pertussis. While the majority of these  
10 results have been generated using PT mutants secreted by B. paraptussis, it is evident that equivalent products are obtained by genetic manipulation of B. pertussis itself. This disclosure, therefore, presents a number of detoxified immunogenic forms of pertussis toxin that would be candidates for inclusion in a novel pertussis vaccine, and a method for producing them in B. pertussis.

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### EXAMPLES

20 Methods of molecular genetics, protein biochemistry and fermentation and hydridoma technology used but not explicitly described in this disclosure and these Examples are amply reported in the scientific literature and are well within the ability of those skilled in the art.

#### Example I:

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This Example illustrates the preparation and purification of PT.

Culture supernatants of B. pertussis (strain 10536) were concentrated 20-50 times by ultrafiltration through a 10,000 or 20,000 molecular weight cut-off membrane using a Millipore Pellicon cassette system. The toxin was adsorbed from crude concentrates by passage through a fetuin-agarose affinity column  
30 equilibrated with 1 M potassium phosphate, 10 mM NaCl at pH 7.5. The volume of adsorbent was typically 1 ml per mg of toxin. The loaded column was washed with 100 mM potassium phosphate, 1 M NaCl at pH 7.5, then eluted with the same buffer containing 3 M potassium thiocyanate to desorb the toxin. Pooled fractions were dialyzed against 50 mM Tris-HCl, 200 mM NaCl containing 10% v/v glycerol at pH 8.0, to remove thiocyanate, then against 50 mM Tris-HCl, 200 mM NaCl containing 50% v/v glycerol at pH 8.0, to  
35 allow storage of the product at -200C. The yield as determined by ELISA was typically 90-95%. The purity as determined by SDS-PAGE was 90-95%, the major contaminant being FHA. For further purification the stored toxin was diluted five-fold with water and loaded onto a hydroxyapatite column of volume 1 ml per mg of toxin, that had been equilibrated with 10 mM potassium phosphate at pH 8.0. The column was washed with 30 mM potassium phosphate at pH 8.0 then eluted with 100 or 200 mM potassium phosphate  
40 to desorb the toxin. Pooled fractions were dialyzed against 100 mM potassium phosphate containing 50% v/v of glycerol at pH 8.0 and the final product stored at The yield was typically 90-95%, and the purity >99% as shown by SDS-PAGE.

#### 45 Example II:

This Example illustrates the preparation of PT subunit S1.

PT was adsorbed to fetuin-agarose as described in Example I, then the column was washed with CHAPS buffer (500 mM urea, 50 mM potassium phosphate, 100 mM NaCl and 1% w/v of CHAPS(3-  
50 cholamidopropyl)-dimethylammonio]-1-propanesulphonate) at pH 7.5). The column was eluted with the same medium containing 500  $\mu$ M of adenosine triphosphate (ATP). The S1 subunit emerged as a sharp peak at the column volume. The pooled fractions were passed through a clean fetuin-agarose column equilibrated with CHAPS/ATP medium to remove residual B oligomer, then dialyzed against 100 mM potassium phosphate containing 50% v/v glycerol at pH 8.0 for storage at -200C. S1 was quantified by  
55 reverse-phase HPLC on a Vydac C4 column by comparison of the integrated peak area with that of a PT standard. The yield was typically only 20-25%, but the product was free of other subunits as demonstrated by both SDS-PAGE and reverse-phase HPLC.

**Example III:**

This Example illustrates the photocrosslinking of NAD to the S1 subunit.

Reaction mixtures (100  $\mu$ l) containing 50  $\mu$ g/ml of S1, 10 mM dithiothreitol and 50  $\mu$ M MAD in CHAPS buffer were placed in the wells of a 96-well microtitre plate set in ice, preincubated for 30 min and then irradiated at 254 nm for periods up to 3 hr at a distance of 5 cm with a 9 W mercury lamp. Samples were then assayed for residual NAD glycohydrolase activity. The enzyme activity of S1 was completely abolished after irradiation for 2 hr, whereas the extent of photoinactivation was only 40% under the same conditions but in the absence of NAD. This result indicated that NAD-dependent photochemical events had occurred.

To discover which part of the NAD molecule interacted with the protein and the extent of crosslinking, S1 was irradiated under identical conditions with [carbonyl- $^{14}$ C]NAD or [adenine- $^{14}$ C]NAD. Aliquots were removed at intervals up to 3 hr and treated with trichloroacetic acid (TCA) to 10% w/v. The precipitated protein was collected by filtration, washed with fresh 10% w/v TCA and counted in a scintillation counter. Results indicated that the radiolabel was incorporated from the nicotinamide moiety rather than from the adenine moiety, and that the extent of incorporation was 0.75 mol label per mol protein.

**Example IV:**

This Example identifies the site of photocrosslinking on the S1 subunit.

Reaction mixtures (3 ml) containing 100  $\mu$ g/ml of S1, 10 mM dithiothreitol and 50  $\mu$ M [carbonyl- $^{14}$ C]-NAD in CHAPS buffer were placed in a Petri dish on ice to give a 1 mm layer, then irradiated at 254 nm for 2 hr with gentle magnetic stirring. The solution was deaerated with nitrogen, further reduced with dithiothreitol and S-alkylated with 4-vinylpyridine to prevent oxidation of thiol groups. The reaction mixture was dialyzed extensively against 10 mM acetic acid and the radiolabelled protein was collected after precipitation with 20% w/v TCA.

The precipitated protein (1 mg) was redissolved in 2 M urea, 200 mM ammonium bicarbonate to 500  $\mu$ g/ml and digested with 50  $\mu$ g/ml trypsin for 20 hr at 37 C. The mixture was acidified and fractionated on a 1x25cm Vydac C<sub>18</sub> reverse-phase HPLC column, using a linear gradient of 0-50% acetonitrile in 10 mM trifluoroacetic acid (TFA). Fractions were checked by scintillation counting, which revealed two major radioactive peptides, denoted A and B, accounting for 50% of the eluted radioactivity. The peptide pool was lyophilized, redissolved in 10 mM TFA, 6 M guanidinium chloride and separated on a Vydac C18 column using a 20-30% acetonitrile gradient in 10 mM TFA. Each peptide was further purified to homogeneity on the same column by applying an acetonitrile gradient in 20 mM ammonium acetate at pH 6.5, and the solutions evaporated to dryness. Their specific radioactivities were consistent with only one labelled site per molecule.

The two peptides were sequenced by automated Edman degradation. A portion of the sequenator effluent was diverted for monitoring of radioactivity. The results are shown in Figure 1. Up to cycle 15, the sequences proved to be identical and correspond unequivocally to residues 118-132 of mature S1. In both peptides radioactivity was associated with an unidentified amino acid released at cycle 12, corresponding to position 129 in mature S1. No radioactivity was detected at cycles beyond 15. Thus it was established that GLU<sup>129</sup> was the site of crosslinking, and is therefore likely to be an important component of the nicotinamide interaction site.

**Example V:**

This Example illustrates the preparation of *B. pertussis* chromosomal DNA.

Two litres of *B. pertussis* (strain 10536) grown in modified Stainer-Scholte medium as 16x125 ml aliquots using a 4 ml inoculum of saturated growth for each flask. This medium consists of L-proline 5g/L, NaCl 2.5 g/L, KH<sub>2</sub>PO<sub>4</sub> 0.5 g/L, KCl 0.2 g/L, MgCl<sub>2</sub>.6H<sub>2</sub>O 0.1 g/L, Tris 1.5 g/L, casamino acids 10 g/L, methyl- $\beta$ -cyclodextrin 2 g/L, CaCl<sub>2</sub>.2H<sub>2</sub>O 0.02 g/L, mono-sodium glutamate 10g/L, L-cysteine 0.004%, FeSO<sub>4</sub>.7H<sub>2</sub>O 0.001%, niacin 0.004%, glutathione 0.015%, and asorbic acid 0.04%, pH.7.6. Samples were grown in 500 ml flasks, on a shaker at 35-36 C, 150 rpm for 16.5 hr to log phase. The cells were spun in 500 ml aliquots at 5000xg for 1 hr at 4 C. Each aliquot was washed with 25 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5,) then resuspended in 20 ml TE and frozen at -70 C. One pellet was resuspended in 90 ml TE and pronase added to 500  $\mu$ g/ml. SDS was added to 1% and the sample incubated at 37 C for 21.5 hr generating a clear lysate. The lysate was extracted with 1 volume of phenol saturated Tris-HCl at pH



7.5 at room temperature for 2 hr, with gentle agitation. The phases were separated by centrifugation at 2800xg for 15 min at 20°C and the aqueous phase extracted similarly with 1 volume of 1:1 phenol:chloroform. The phases were separated by centrifugation at 2100xg for 10 min at 20°C and the aqueous phase extracted with chloroform for 2 hr as described. The phases were separated by centrifugation at 1600xg for 5 min at 20°C and the aqueous phase subjected to dialysis at 4°C against 2 L of 1 M NaCl for 24 hr with one change of buffer, then against 2 L TE for 48 hr with one change of buffer.

#### Example VI:

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This Example illustrates the generation of B. pertussis gene libraries.

##### 1) $\lambda$ gIII EcoR I library

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B. pertussis DNA (10  $\mu$ g) was digested with EcoR I (10 units) in the presence of 100 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 100  $\mu$ g/ml BSA, 1  $\mu$ g/ml RNase A for various lengths of time in order to generate a set of partially digested DNA fragments. At each time point of 0.25, 0.5, 1, 2, 4 and 8 hrs, the sample was placed at 0°C and EDTA added to 20 mM to stop the reaction. The samples were pooled and separated on a 10-40% sucrose gradient in TNE (20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1M NaCl) at 85,000xg for 20 hr at 20°C. The gradient was fractionated from the top as 24 aliquots (0.5 ml) to which 1 ml aliquots of absolute ethanol were added to precipitate the DNA. The samples were incubated on dry ice for 30 min then centrifuged at 12,000xg for 5 min at 4°C. The pellets were washed with 750  $\mu$ l of 70% ethanol, incubated on dry ice for 5 min, centrifuged at 12,000xg for 5 min, then dried. Each pellet was resuspended in 25  $\mu$ l of sterile water and 5  $\mu$ l aliquots of every alternate fraction were submitted to agarose gel electrophoresis to determine the size of the fragments. Samples containing DNA ranging in size from approximately 0.5 kb to 9 kb were pooled. The pooled EcoR I-digested B. pertussis DNA (0.4  $\mu$ g) was ligated with EcoR I-digested, dephosphorylated  $\lambda$  gIII DNA (0.5  $\mu$ g) and was packaged into phage particles using a commercial kit. The phage library was propagated in E. coli Y1090 cells and was titred at approximately 10<sup>10</sup> plaque-forming units(pfu)/ $\mu$ g of  $\lambda$  gIII DNA. The library was amplified to 4x10<sup>10</sup> pfu/ml for screening clones. The amplification was performed on plates by growing cells to saturation overnight in media containing 0.2% maltose, then adding 10<sup>4</sup> to 10<sup>5</sup> pfu of library per 0.6 ml of cells and allowing the phage to adsorb to the cells for 15 min at 37°C. The sample was mixed with soft agar, plated, and incubated overnight at 37°C. The soft agar/cells/phage layer was scraped from the confluent plates which were washed with 4 ml SMG buffer (0.4 M NaCl, 10 mM MgSO<sub>4</sub>, 50 mM Tris-HCl, pH 7.5, 0.01% gelatin). The wash and phage agar were combined, 100  $\mu$ l of chloroform added, and the mixture incubated at 37°C for 15 min with gentle agitation. The sample was centrifuged at 4000xg at 4°C for 10 min twice to obtain a clear supernatant. Chloroform was added to a final concentration of 0.3% and the library stored at 4°C.

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##### 2) $\lambda$ Charon 35 Sau3A I library

B. pertussis DNA (3x166  $\mu$ g) was digested with Sau3A I (3x220 units) in the presence of 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 100  $\mu$ g/ml BSA for 1 min, 2 min, or 3 min in order to generate very large fragments of DNA. After each reaction, EDTA was added to 20 mM and then 2.5 volumes of absolute ethanol added to precipitate the DNA as described above. The DNA was resuspended in TNE and separated on a 10-30% sucrose in gradient in TNE as described above. Fractions were taken as before and the DNA fragment sizes visualized by agarose gel electrophoresis.  $\lambda$  Charon 35 DNA (2x50  $\mu$ g) was ligated to generate a circularized form before being digested with BamH I (2x20 units) in the presence of 150 mM NaCl, 6 mM Tris-HCl pH 7.9, 6 mM MgCl<sub>2</sub>, 100  $\mu$ g/ml BSA to remove the stuffer fragments. The lambda arms were purified by pelleting through an 8-20% potassium acetate gradient at 85,000xg, for 16 hr at 32°C. The Sau3A I digested DNA was ligated with the lambda arms at 6°C for 72 hr, then packaged into phage using a commercial kit. The phage library was propagated in E. coli LE392 cells and was titred at approximately 1x10<sup>5</sup> pfu/ $\mu$ g of lambda arms. The library was amplified to 1-2x10<sup>10</sup> pfu/ml for screening as described above.

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#### Example VII:

This Example illustrates the screening of the B. pertussis libraries.

#### 1) $\lambda$ gtlI genomic library

A 30-base oligonucleotide probe was synthesized based on the nucleotide sequence of the gene encoding PT subunit S4. The DNA was purified from urea/acrylamide gels by uv-imaging and anion exchange chromatography on Whatman cellulose DE52. The sequence of the oligonucleotide was 5'GTAGCCATGAAGCCGTATGAAGTCACCCCG3', coding for amino acids 16-25 of the mature S4 protein. The oligonucleotide was 5' end-labelled in a reaction mix containing 10  $\mu$ g DNA, 25  $\mu$ Ci [8-<sup>32</sup>P]ATP, 4 units polynucleotide kinase in the presence of 50 mM Tris-HCl, pH 9.5, 10 mM MgCl<sub>2</sub>, 5 mM DTT, 5% glycerol by incubation at 37°C for 15 min. ATP was added to 1.5 mM and the incubation continued for 1.75 hr at 37°C. 10  $\mu$ g of tRNA were added as carrier and the labelled DNA was separated from free ATP on a Sephadex G50 superfine column eluted with 0.1 M triethylammonium bicarbonate, pH 7.6. Peak fractions were pooled and lyophilized to dryness. The pellet was washed with sterile water, resuspended and then resuspended at approximately 0.1  $\mu$ g/ $\mu$ l.

Aliquots of the  $\lambda$  gtlI B. pertussis genomic library were plated on a Y1090 lawn on NZCYM plates containing 0.2% maltose. Plaque-lifts were made onto nitrocellulose filters which were sequentially treated with denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 1 min, neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl pH 8.0) for 5 min, and rinsed briefly in 2xSSPE (0.36 M NaCl, 20 mM sodium phosphate, pH 7.4, 2 mM EDTA) before being baked at 80°C under vacuum for 2 hr to fix the DNA. Nitrocellulose filters were subsequently incubated in a prehybridization buffer comprising 5xSSC (0.75 M NaCl, 75 mM sodium citrate, pH 7.5), 5x Denhardt's mixture (0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% BSA), 0.1% SDS, 100  $\mu$ g/ml herring sperm DNA for 2 hr at 45°C. The prehybridization buffer was removed and fresh buffer containing 10<sup>7</sup> cpm of [<sup>32</sup>P]-labelled oligonucleotide probe was added. Hybridization was carried out at 45°C for 16 hr. The radioactive solution was removed and the filters rinsed briefly twice at room temperature with 5xSSC, 0.1% SDS to remove unbound probe. The filters were further washed twice with 5xSSC, 0.1% SDS for 1 hr at 50°C then air-dried and subjected to autoradiography.

The plaque-containing plates were aligned with their autoradiograms and putative positive plaques were subjected to another two rounds of purification on plates. One clone ( $\lambda$  gtlI-15-4-1) was chosen for detailed examination by Southern blot analysis.

#### 2) $\lambda$ Charon 35 genomic library

Aliquots of the  $\lambda$  Charon 35 B. pertussis genomic library were plated on an LE392 lawn on NZCYM plates containing 0.2% maltose. The plaque-lift, hybridization and washing protocols were performed as described. Positive plaques were purified twice more on plates and several clones,  $\lambda$  Ch 35 111, 121, 411, 421 and 431, were examined by Southern blot analysis.

### Example VIII:

This Example illustrates the analysis of the genomic clones.

#### 1) Preparation of phage DNA

One litre (2x500 ml) of phage culture was prepared. LE392 or Y1090 cells were grown overnight in medium containing 0.2% maltose. Cells (10<sup>10</sup>) were spun down at 4400xg for 5 min at 4°C and the pellet resuspended in 1 ml SMG buffer. Phage stock (1.2x10<sup>8</sup> pfu) was added to the mixture and incubated at 37°C for 15 min to absorb the phage to the cells. The phage/cell mixture was inoculated into 500ml of medium and the culture shaken vigorously at 37°C until lysis began (4-4.5 hr). Chloroform (10ml) was added and shaking continued at 37°C for an additional 15 min to complete the lysis. The sample was cooled to room temperature and DNase I and DNase-free RNase A (1  $\mu$ g/ml each) were added for 30 min at room temperature. The cell debris was pelleted at 3500xg for 20 min, then 29.2 g NaCl and 50 g polyethylene glycol (PEG 6000) were added to 500 ml of supernatant. The samples was gently agitated at room temperature to dissolve the solids, then incubated at 0°C for 1-2 hr to precipitate the phage. The

phage were harvested by centrifuging at 4400xg at 4°C for 20 min and were resuspended in 8 ml TM buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgSO<sub>4</sub>). Extraction with 8 ml chloroform to remove the PEG gave a clear supernatant which was applied to a step gradient of 5% and 40% glycerol in TM buffer and centrifuged at 154,000xg at 4°C for 1 hr. The supernatant was discarded leaving a phage pellet which was resuspended in 0.5 ml TM buffer. DNase I was added to 5 µg/ml and RNase A to 50 µg/ml and the sample incubated at 37°C for 30 min. EDTA was added to 20 mM, pronase to 0.5 mg/ml, SDS to 0.5%, and the sample further incubated at 37°C for 1 hr. The sample was gently extracted once each with phenol, phenol:chloroform 1:1, and chloroform and the phage DNA precipitated with ethanol.

## 2) Results

Clone 15-4-1 which was derived from the EcoR I gtlI library, was found by Southern blot analysis to contain the 4.6 kb EcoR I fragment encoding the entire TOX gene plus small 5' - and 3' -flanking regions.

The λ Charon 35 clones were found to be closely related. Some clones contained the entire TOX operon plus flanking regions in either orientation, and others did not include the entire TOX region.

The maps of clones 15-4-1, Ch 111, Ch 121/411, Ch 431 and Ch 421 are shown in Figure 2.

## Example IX:

This Example illustrates the construction of pUC-based plasmids containing the pertussis toxin operon (TOX) or portions thereof.

Phage DNA from the λ gtlI clone 15-4-1 was prepared as described and digested with restriction endonuclease EcoR I using standard methods. The DNA was purified by gel electrophoresis in low-melting-point agarose. The 4.6 kb band was identified by uv-illumination of the ethidium bromide stained gel and excised. The DNA was extracted by a freeze-thaw technique employing 0.3 M sodium acetate, pH 7.0 and was precipitated with ethanol. DNA from pUC8:2, a derivative of pUC8 containing two extra restriction sites for Bgl II and Xba I in its multiple cloning site, was digested with EcoR I. The linearized DNA was dephosphorylated by standard methods using calf alkaline phosphatase (CAP), phenol extracted and precipitated with ethanol.

The pUC8:2-vector DNA and 15-4-1-derived-TOX DNA were ligated in a standard reaction and the ligation mixture used to transform competent JM109 cells according to standard procedures. The resulting colonies were analysed by a rapid DNA screening technique and two clones were chosen for large-scale preparation of plasmid DNA. These clones, J-169-1 and J-169-2, differed only in the orientation of the TOX insert. The construction of these clones is illustrated in Figure 3.

## Example X:

This Example illustrates the sequencing of the TOX operon.

### 1) Clones used

The clone J-169-1 was used as the source for all sequencing clones. The TOX operon was divided into five approximately equal DNA segments and was subcloned into M13mp18, M13mp19 or pUC8:2 as illustrated in Figures 4a, b, c, d and e.

### 2) Preparation of samples

M13 clones were maintained in JM101 and DNA for sequencing was prepared from single plaques on homogeneous plates. A saturated JM101 culture was diluted 1:50 with fresh medium and infected with a single plaque. The culture was grown with vigorous shaking at 37°C for 6 hr. The cells were removed by centrifugation and the supernatant treated with 1/4 volume of 20% PEG 6000, 2.5 M NaCl to precipitate phage. The suspension was centrifuged and the phage pellet was resuspended in TE, then extracted gently twice each with phenol, phenol:chloroform (1:1) and chloroform. The phage DNA was precipitated with

sodium acetate and ethanol, washed with 70% ethanol and dried. The DNA was resuspended in sterile water to a concentration of about 1 µg/ml for sequencing.

Sequencing primers of approximately 17-20 bases were synthesized on an ABI 380A DNA synthesizer using phosphoroamidite chemistry and were purified as described above.

### 3) Sequencing

The dideoxy chain termination method of Sanger was used for all sequencing reactions, employing either Klenow polymerase or Sequenase T7 enzyme.

### 4) Results

The entire TOX operon, as previously defined, was sequenced and the result compared with published sequences. There was excellent agreement with the TOX sequence of strain BP 165 reported by Nicosia et al., except for four base differences. The T at position 315 in the 5'-flanking region is unique to B. pertussis strain 10536. The three other substitutions are in the S1-coding region at positions 710, 1200 and 1202 resulting in two unique amino acids, GLU34 and VAL198. The nucleotide sequence and derived amino acid sequence are shown in Figure 5.

#### Example XI:

This Example illustrates mutagenesis of the TOX gene.

#### 1) Clones used

For mutations in the S1 gene, clone S-2403 (M13mp18/S1) was used and for the mutations in the S3 gene, clone S-2664-5-6, (M13mp18/S3(c)) was used. These clones are represented in Figure 4.

#### 2) Mutagenesis protocol

Single-stranded DNA was prepared from phage stocks derived from single plaques on homogeneous plates as described previously. Mutagenic primers of appropriate sequence and length were synthesized on an ABI 380A DNA synthesizer.

Commercial kits based on the phosphorothioate procedure developed by Eckstein were used for in vitro mutagenesis. Briefly, the mutagenic oligonucleotide was annealed to the single-stranded (wild-type) template and polymerization carried out using as substrates a phosphorothioate dCTP analogue and natural dATP, dGTP and dTTP. The double-stranded DNA was nicked with Nci I and the native strand digested with exonuclease III beyond the point of the mutation. The complementary strand was protected from Nci I-nicking by the phosphorothioate groups. The complementary strand then served as a template in a second round of polymerization to yield double-stranded DNA with the mutation in both strands. This DNA was amplified in E. coli, and the mutation confirmed by sequencing.

Thirty-five primary mutations were generated and an additional 14 were derived by constructing crosses among these. The mutation efficiency varied with the change desired. From one to six base changes and deletions of up to 15 consecutive bases were accomplished. The resulting amino acid changes are summarized in Table 1a.

#### Example XII:

This Example describes the construction of plasmids for expression of mutated TOX genes in B. parapertussis and characterization of the PT analogues produced.

## 1) Replicating plasmids

Replicative-form DNA from M13 clones was used to reconstruct the TOX operon containing the desired mutation in pRK404. pRK404 is a derivative of pRK290, a conjugating plasmid of the pRK2 family, incompatibility group P-1. It is 10.6 kb in size, carries a tetracycline resistance ( $Tet^R$ ) gene, and has a multiple cloning site from pUC8. The construction schemes for reintegrating S1 and S3 primary mutations into the operon are shown in Figure 6 and the resulting clones are indicated in Table 1a. Crossed mutations in S1 were generated using internal restriction sites, especially the unique Sal I site. A general scheme for crossed mutations in S1 is also shown in Figure 6 and the resulting clones are indicated in Table 1a.

## 2) Suicide plasmids

A conjugative but non-replicative plasmid was developed for random integration of TOX or mutated TOX into the chromosome of *Bordetella* species. Figure 7 demonstrates the construction of these clones.

Plasmids of the types described in (1) and (2) above were introduced into *B. pertussis* by conjugation. The resulting strains were grown in shake-flasks or in a fermentor, and the culture supernatants were assayed as follows for concentration of toxin analogue by ELISA. Microtitre plates were coated with fetuin (2  $\mu$ g/ml) in 0.05 M potassium carbonate, pH 9.6 at 4 °C overnight in a humid environment. The plates were then washed twice with Delbecco's PBS containing 0.1% w/v Tween-20 and dried. Sample supernatants or wild-type PT were serially diluted and added to the wells, and the plates incubated for 30 min at room temperature then washed. Bound PT was detected using peroxidase-conjugated affinity-purified rabbit anti-PT antibodies.

Residual toxicity was measured by the CHO cell clustering assay, to determine the toxicity relative to native PT. Certain PT mutants were purified as described for native PT in Example I, and assayed for ADP-ribosyltransferase activity. These data are summarized in Table 1b. Expression of the S1 epitope recognized by MAb PS21 was assessed by a modified indirect ELISA on culture supernatants. Fetuin-bound PT analogues were reacted with PS21 as the first antibody and visualized with an enzyme-conjugated affinity-purified goat anti-mouse IgG as the second antibody. The presence or absence of the S1 epitope recognized by MAb PS21 is indicated in Table 1b.

## Example XIII:

This Example illustrates the construction of plasmids for deletion and replacement of the endogenous *B. pertussis* TOX operon.

## 1) Plasmids containing TOX flanking regions

## a) 5'-flanking region

The Ch 421 DNA was first digested with Bgl II and an 11 kb fragment was purified by agarose gel electrophoresis. The Bgl II fragment was digested with Xma I and the 5 kb band subcloned into pUC8:2 previously restricted with Xma I and dephosphorylated. JM109 cells were transformed with the ligation mixture to give colonies which were analysed by a rapid DNA screening method. The clone J-183-9 was found to contain approximately 2.9 kb of the 5'-flanking region, the TOX promoter and the genes for subunits S1 and S2. Figure 8a shows the derivation of clone J-183-9.

## b) 3'-flanking region

The Ch 111 DNA was digested with Sal I and an approximately 8 kb fragment of *B. pertussis* DNA was gel-purified. This DNA fragment was inserted into pUC8:2 previously digested with Sal I and dephosphorylated. JM109 transformants were screened and the clone J-219-111-3 was identified as containing part of the S1 gene, all of the remaining structural genes, and about 3.9 kb of the 3' flanking region. Figure 8b shows the construction of this clone.

c) TOX gene with 5' - and 3' -flanking regions.

Clone J-183-9 was digested with Xba I and the approximately 7 kb fragment containing pUC8:2, the 5' - flanking region and the promoter region of the S1 gene was gel-purified and dephosphorylated. J-219-111-3 DNA was digested with Xba I and the approximately 8 kb fragment containing the structural genes for subunits S2 to S5 and the 3' -flanking regions was gel-purified. These DNA fragments were ligated and the JM109 transformants were screened to give clone J-229-17. This clone contains about 2.9 kb of the 5' - flanking sequence, the entire TOX operon, and about 4 kb of the 3' -flanking sequence. Its construction is illustrated in Figure 8c.

## 2) TOX-deleting plasmids

Plasmid S-2832-5 contains the Tet<sup>R</sup> gene from plasmid pRK404 and its construction is shown in Figure 9. The Tet<sup>R</sup> gene was cloned as an EcoR I/BamH I restriction fragment into plasmid pN01523 to generate pGZ62. Plasmid pGZ63 contains the 5' - and 3' -flanking regions without any intervening DNA. The S12-Tet<sup>R</sup> gene-sandwich from pGZ62 was cloned between the flanking regions of pGZ63 to produce plasmid pGZ65. The construction of these plasmids is summarized in Figure 8d.

## 3) TOX-reintegrating plasmids

To express mutated TOX genes in TOX<sup>-</sup> strains of B. pertussis conjugative suicide plasmids of the type shown in Figure 10 were constructed. They contain the TOX gene, extensive 5' - and 3' -flanking sequences and have a Tet<sup>R</sup> gene for selection cloned downstream from the TOX coding regions.

### Example XIV:

This Example illustrates the deletion of the TOX gene from the B. pertussis chromosome and the reintegration of in vitro-mutated TOX genes.

#### 1). Transformation of B. pertussis

Strains of B. pertussis were transformed by electroporation. Cells were grown in 100 ml of modified Stainer-Scholte medium to a density of about 109 cells/ml, harvested in a clinical centrifuge (4000xg for 15 min at 20 °C), washed in 25 ml of electroporation buffer (0.3 M sucrose, 1 mM MgCl<sub>2</sub>, 7 mM potassium phosphate, pH 7.2) and resuspended in 10 ml of the same. Plasmid DNA was added to 500 µl of the cell suspension and the mixture incubated on ice for 10 min. The cells were subjected to a single 25 kV/cm, 40 µs exponential decay voltage pulse with a BTX Transfector 100, using a cuvette electrode with a 0.8 mm gap. Three ml of medium were added and the cells incubated with shaking at 37 °C for 60 min. The cells were harvested by centrifugation at 12,000xg for 2 min, resuspended in 100 µl of medium, spread onto a Bordet-Gengou plate with antibiotic selection and incubated for 2-5 days at 37 °C.

#### a) Deletion and replacement of the TOX operon

B. pertussis str29 is a spontaneous rpsL streptomycin resistant derivative of B. pertussis 10536. Plasmid pGZ65 contains a gene cartridge consisting of the pRK404 Tet<sup>R</sup> gene and the E. coli S12 gene cloned between the 5' - and 3' -flanking sequences of the TOX operon. This plasmid was linearized with Hind III and used to transform B. pertussis str29 to Tet<sup>R</sup>, Str<sup>S</sup> resulting in the deletion of the TOX operon by homologous recombination. This TOX-deleted strain was termed 29-8. To excise the S12-Tet<sup>R</sup> gene cartridge, strain 29-8 was subsequently transformed with linear pGZ63 plasmid DNA. Plasmid pGZ63 consists of the TOX 5' - and 3' -flanking sequences but contains no intervening DNA. Transformation with this plasmid resulted in the generation of B. pertussis 29-9 which is a streptomycin-resistant, TOX-deleted strain but contains no heterologous DNA inserted at the TOX locus. This strain was used as the host for expression of in vitro mutated TOX genes. Plasmids of the type shown in Figure 10 contain a gene

cartridge consisting of a mutated TOX gene and a Tet<sup>R</sup> gene. This gene cartridge was recombined into the B. pertussis 29-9 chromosome following introduction of the plasmid into the strain by conjugation or transformation. Expression of the TOX gene, toxicity of the PT analogues and maintenance of the S1 epitope recognised by MAb PS21 were determined as described before. The recombinant B. pertussis strains constructed and the properties of the secreted PT analogues are shown in Table 5.

#### Example XV:

This Example describes the in vivo testing of PT mutants in mice.

Pt mutants were purified from culture supernatants and recombinant B. parapertussis strains as indicated in Example I. These proteins were injected into mice at three different doses to test the following characteristics, according to standard procedures: acute toxicity, histamine sensitization activity and potency in the mouse intracerebral challenge test. The results are presented in Table 2.

To test their immunogenicity, PT analogues were injected into female BALB/C mice, 9 to 11 weeks old, at doses of 2.0, 0.5 and 0.125 µg. Mice were pre-bled and immunized on day 0. On day 23 the mice were bled again and boosted with the same immunogen, and on day 37 the mice were bled again. Blood samples (0.4 - 0.5 ml/mouse) were collected by orbital sinus bleeding and the resulting sera stored at -20°C to await testing. Sera were assayed for their ability to neutralize PT-induced CHO cell clustering (Table 3), and for specific antibody responses in antigen-coat, indirect ELISA (Table 4). As may be seen from Tables 3 and 4, PT analogues are capable of inducing neutralizing antibodies and pool anti-PT, anti-S1 and anti-B oligomer responses.

#### SUMMARY OF DISCLOSURE

In summary of this disclosure, the present invention provides a novel method of detoxifying pertussis by identification of specific functional sites of pertussis toxin and production of recombinant holotoxins by site-directed mutagenesis of the toxin gene. The resulting toxin analogues are detoxified, retain an immunodominant S1 epitope, are immunogenic and are protective against the disease pertussis. Modifications are possible within the scope of this invention.

TABLE 1a

## Summary of Mutations introduced into Pertussis Toxin

Mutation Number	Mutation	Clone No.
1.	ARG <sup>9</sup> -> Δ <sup>9</sup>	S-2679-1-11
2.	" -> GLU <sup>9</sup>	S-2815-1-8
3.	" -> LYS <sup>9</sup>	S-2953-21
4.	" -> HIS <sup>9</sup>	S-3046-4
5.	ARG <sup>13</sup> -> Δ <sup>13</sup>	S-2679-2-1
6.	" -> GLU <sup>13</sup>	S-2779-2-1
7.	ARG <sup>9</sup> -ARG <sup>13</sup> -> Δ <sup>9-13</sup>	S-2829-2-19
8.	ARG <sup>9</sup> ARG <sup>13</sup> -> GLU <sup>9</sup> GLU <sup>13</sup>	S-2779-3-2
9.	ARG <sup>58</sup> -> GLY <sup>58</sup>	J-444-2-2
10.	ARG <sup>57</sup> ARG <sup>58</sup> -> Δ <sup>57</sup> Δ <sup>58</sup>	J-482-11
11.	TYR <sup>26</sup> -> ALA <sup>26</sup>	S-3123-2
12.	" -> CYS <sup>26</sup>	S-3140-22
13.	CYS <sup>41</sup> -> ALA <sup>41</sup>	S-2515-5-10
14.	" -> SER <sup>41</sup>	S-3124-6
15.	CYS <sup>201</sup> -> ALA <sup>201</sup>	S-2679-3-4
16.	GLU <sup>129</sup> -> Δ <sup>129</sup>	S-2589-6
17.	" -> GLY <sup>129</sup>	S-2515-3-6
18.	" -> GLN <sup>129</sup>	S-2515-1-2
19.	" -> ASP <sup>129</sup>	S-2515-2-4
20.	" -> ASN <sup>129</sup>	S-2852-1-18
21.	" -> LYS <sup>129</sup>	S-2515-4-11
22.	" -> ARG <sup>129</sup>	M-32-2-4
23.	" -> HIS <sup>129</sup>	S-2937-1-2
24.	" -> PRO <sup>129</sup>	S-2959-2-28
25.	" -> CYS <sup>129</sup>	J-478-5
26.	" -> GLY <sup>129</sup> II	J-418-1
27.	" -> GLN <sup>129</sup> II	J-412-9
28.	TYR <sup>130</sup> -> Δ <sup>130</sup>	S-2852-2-1
29.	" -> PHE <sup>130</sup>	S-2836-15
30.	GLU <sup>129</sup> TYR <sup>130</sup> -> GLY <sup>129</sup> ALA <sup>130</sup>	S-2679-4-3
31.	GLU <sup>129</sup> TYR <sup>130</sup> -> GLN <sup>129</sup> ALA <sup>130</sup>	M-38-1
32.	GLU <sup>129</sup> TYR <sup>130</sup> -> GLY <sup>129</sup> PHE <sup>130</sup>	J-444-1-6

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TABLE 1a (con't)

Mutation Number	Mutation	Clone No.
33.	(S3) LYS10 -> GLN10	S-2995-1-2
34.	(S3) TYR92 LYS93 -> ASN92 ARG93	S-2995-2-1
35.	(S3) LYS108 -> ASN05	S-2995-3-1
36.	CYS41 CYS201 -> ALA41 ALA201	S-2818-1
37.	CYS41 GLU129 -> ALA41 GLY129	S-2549-2
38.	ARG9 GLU129 -> GLU9 GLY129 II	S-2966-1-5
39.	ARG9 GLU129 -> GLU9 GLN129 II	S-2967-1-1
40.	ARG9 GLU129 -> GLU9 ARG129	M-45-1
41.	ARG9 GLU129 TYR130 -> GLU9 GLY129 ALA130	S-2956-1
42.	ARG13 GLU129 -> GLU13 GLY129 II	S-2966-2-13
43.	ARG13 GLU129 -> GLU13 GLN129 II	S-2967-2-17
44.	ARG13 GLU129 TYR130 -> GLU13 GLY129 ALA130	S-2961-1
45.	ARG9 GLU129 -> Δ9 GLN129	S-2730-1-1
46.	ARG9 GLU129 TYR130 -> Δ9 GLY129 ALA130	S-2730-3-2
47.	ARG13 GLU129 -> Δ13 GLN129	S-2730-2-1
48.	ARG13 GLU129 TYR130 -> Δ13 GLY129 ALA130	S-2730-4-1
49.	GLU129 -> GLY129	S-3050-1
	(S3) TYR92 LYS93 -> (S3) ASN92 ARG93	
50.	Wild Type	S-2505-4-5

25 Amino acid numbering corresponds to positions in the native subunits (Figure 5)

All mutations are in subunit S1 unless specified as being in S3 (S3)

30 II denotes use of an alternative codon

Δ denotes deleted residue(s)

35 Wild type refers to PT expressed from the unmutated TOX operon in *E. PARAPORTUENSIS*.

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TABLE 1b

In vitro characterization of pertussis toxin analogues  
obtained from recombinant *B. pertussis*.

Mutation Number	Residual Toxicity	ADPR Activity	S1 Epitope
1.	0.2	ND	-
2.	0.1	0.3	+/-
3.	0.1	ND	++++
4.	0.2	0.1	+++
5.	0.3	ND	-
6.	5.0	ND	++++
7.	0.4	0.1	-
8.	0.1	0.9	-
9.	0.7	0.6	+++
10.	0.4	ND	-
11.	0.5	ND	+
12.	6.0	ND	ND
13.	0.3	0.4	-
14.	1.4	ND	ND
15.	0.2	0.1	-
16.	0.1	ND	++
17.	0.1	0.3	++++
18.	0.02	0.1	+/-
19.	0.7	2.5	++
20.	0.1	0.3	++
21.	0.3	0.2	-
22.	0.1	ND	-
23.	0.2	ND	-
24.	0.2	ND	+
25.	0.4	ND	-
26.	0.1	0.3	++++
27.	0.02	0.1	+/-
28.	0.2	0.1	-
29.	12.0	ND	++++
30.	0.2	0.6	-
31.	0.4	ND	-
32.	1.0	ND	++++

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TABLE 1b (con't)

	Mutation Number	Residual Toxicity	ADPR Activity	S1 Epitope
5	33.	100	ND	+++++
	34.	50	100	++++
	35.	20	ND	++++
10	36.	0.2	0.1	-
	37.	0.1	0.1	-
	38.	0.1	0.1	-
	39.	0.1	ND	-
	40.	0.1	ND	-
	41.	0.2	ND	-
15	42.	0.5	ND	-
	43.	3.0	ND	-
	44.	0.3	ND	-
	45.	0.4	ND	-
	46.	0.2	0.1	-
20	47.	0.5	ND	-
	48.	0.4	0.3	-
	49.	0.2	0.1	++++
	50.	100	100	+++++

Residual toxicity is the ratio of the apparent PT concentration determined by the CHO cell clustering assay to the actual concentration of PT mutant determined by ELISA expressed as a percentage.

ADPR activity is the extent of ADP-ribosylation of bovine transducin catalysed by a PT analogue, relative to that catalysed by an equal concentration of wild-type PT, expressed as a percentage.

S1 epitope refers to the expression of an immunodominant S1 epitope recognized by a specific monoclonal antibody PS21, as compared with the wild-type PT (+++++).

ND denotes not determined.

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TABLE 2

Biological Activity of PT mutants in mice			
Analogue	Acute Toxicity LD <sub>50</sub> (ug)	HS Activity LD <sub>50</sub> (ug)	M.P.T. ED <sub>50</sub> (ug)
Native	~2	0.2	≥2
GLY <sup>129</sup>	>5	~3	~2
GLN <sup>129</sup>	>16	>3	16
ASN <sup>129</sup>	>5	~3	1.5
GLU <sup>58</sup>	>5	1.5	8.5
GLY <sup>129</sup> (S3)ASN <sup>92</sup> ARG <sup>93</sup>	>20	7	2
(S3)ASN <sup>92</sup> ARG <sup>93</sup>	3	0.4	≥2
<p>HS Activity denotes histamine sensitizing activity.</p> <p>M.P.T. denotes mouse intracerebral challenge protection test.</p> <p>LD<sub>50</sub> is the dose resulting in death of 50% of the test animals.</p> <p>ED<sub>50</sub> is the dose resulting in protection of 50% of the test animals.</p> <p>Native denotes PT from <u>B. pertussis</u> 10536.</p>			

TABLE 3

Neutralizing effect of immune sera on PT-induced CHO cell clustering			
Analogue Dose (ug)	Pre-bleed	Post-1 bleed	Post-2 bleed
GLY <sup>129</sup>			
2.0	<2	<2	256
0.5	<2	<2	128
0.125	<2	<2	64
GLN <sup>129</sup>			
2.0	<2	<2	128
0.5	<2	<2	256
0.125	<2	<2	128
ASN <sup>129</sup>			
2.0	<2	<2	512
0.5	<2	<2	128
0.125	<2	<2	256
Saline	<2	<2	<2
<p>Mice were pre-bled and immunized on day 0. On day 23 they were bled again (post-1 bleed) and boosted. Final sera were obtained on day 37 (post-2 bleed).</p> <p>The neutralizing ability of the sera is expressed as the maximum dilution at which CHO cell clustering was inhibited.</p>			

TABLE 4

Specific antibody titres of immune sera									
Analogue Dose (ug)	Pre-bleed			Post-1 bleed			Post-2 bleed		
	PT	S1	B	PT	S1	B	PT	S1	B
GLY <sup>129</sup>									
2.0	NR	NR	NR	63	2	50	500	80	200
0.5	NR	NR	NR	13	1	8	160	32	56
0.125	NR	NR	NR	10	0.5	8	200	32	80
GLN <sup>129</sup>									
2.0	NR	NR	NR	22	0.7	20	200	40	125
0.5	NR	NR	NR	8	0.5	6	200	40	100
0.125	NR	NR	NR	5	<0.5	2	125	20	50
ASN <sup>129</sup>									
2.0	NR	NR	NR	40	1	40	500	140	280
0.5	NR	NR	NR	7	<0.5	3	316	22	80
0.125	NR	NR	NR	7	<0.5	4	180	63	125
Saline	NR	NR	NR	NR	NR	NR	NR	NR	NR
Immunization and bleeding were performed as described in Table 3. Antigens used were PT holotoxin, isolated S1 subunit and isolated B oligomer. The units are the dilution factor divided by 1000 giving an ELISA absorbance value equal to twice the background. NR denotes not reactive with antigen.									

TABLE 5

In vitro characterization of pertussis toxin analogues from recombinant B. pertussis				
Mutation Number	Clone	Residual Toxicity	ADPR Activity	S1 Epitope
9	S-3036-2	0.2	0.3	+++
13	S-3122-3-1	0.1	ND	ND
17	S-2962-1-2	0.2	ND	ND
18	S-2962-2-1	0.1	ND	ND
34	S-3122-3-1	50	ND	+++++
49	S-3122-2-3	0.1	ND	+++
50	S-3006-3	100	100	+++++
All terms are as defined in Tables 1a and 1b. ND denotes not determined.				

## Claims

1. An immunoprotective, genetically-detoxified mutant of pertussis toxin.
2. The mutant claimed in claim 1 characterized by genetic modification of the A portion (S1 subunit) of the toxin, the B portion of the toxin or both the A and B portions of the toxin.
3. The mutant claimed in claim 1 or 2, characterized in that a single amino acid in the native pertussis toxin is removed or replaced.
4. The mutant claimed in claim 3, characterized in that the single amino acid is GLU<sup>129</sup> and is removed entirely or is replaced by GLY<sup>129</sup>, or is ARG<sup>58</sup> and is replaced by GLU<sup>58</sup>.
5. The mutant claimed in claim 1 or 2, characterized in that multiple amino acids in the native pertussis toxin are removed or replaced.
6. The mutant claimed in claim 5, characterized in that the multiple amino acids are GLU<sup>129</sup> TYR<sup>130</sup> and are replaced by GLY<sup>129</sup> PHE<sup>130</sup>, or are GLU<sup>129</sup>/(S3)TYR<sup>92</sup>LYS<sup>93</sup> and are replaced by GLY<sup>129</sup>/(S3)-ASN<sup>92</sup>ARG<sup>93</sup>.
7. The mutant claimed in any one of claims 1 to 6, characterized by a residual toxicity less than about 0.5% of the toxicity of the native toxin.
8. The mutant claimed in any one of claims 1 to 7, characterized in that the mutant is produced by site-directed mutagenesis of the native pertussis toxin gene.
9. The mutant claimed in any one of claims 1 to 8, characterized in that the mutant possesses a decreased histamine sensitivity activity.
10. A vaccine against Bordetella pertussis, characterized by an effective amount of a mutant as claimed in any one of claims 1 to 9, or a toxoid thereof, and a physiologically-acceptable carrier therefor.
11. A conjugate vaccine, characterized by an effective amount of an active conjugate comprising a mutant as claimed in any one of claims 1 to 9 covalently bonded to a hapten, polysaccharide or polypeptide to elicit an antibody response to the hapten, polysaccharide or polypeptide.
12. A strain of Bordetella pertussis characterized by the absence of the toxin operon and foreign DNA and by the ability to be grown in the absence of antibiotics to produce B. pertussis antigens free of pertussis toxin.
13. A strain of Bordetella pertussis characterized by the toxin operon having been replaced by a mutant gene formed by site-directed mutagenesis of at least one specific amino acid residue responsible for pertussis toxin toxicity.
14. The strain claimed in claim 13, characterized in that the mutant gene comprises a clone numbered S-3036-2, S-3122-3-1, S-2962-1-1, S-2962-2-1 or S-3122-2-3.
15. A native Bordetella pertussis 10536 TOX operon having the nucleotide sequence and structural gene translation shown in Figure 5.
16. A method for the production of an immunoprotective, genetically-detoxified mutant of pertussis toxin, characterized by the steps of (a) identifying at least one amino acid residue of the toxin which confers toxicity to the toxin; (b) effecting site-directed mutagenesis of the toxin gene to remove or replace said at least one such residue and produce a mutated toxin operon; (c) substituting the mutated toxin operon for the native gene in the Bordetella pertussis organism; and (d) growing the transformed organism to produce immunoprotective, genetically-detoxified toxin.
17. The method claimed in claim 16, characterized in that the at least one amino acid residue is identified as a single functionally-active amino acid in the S1 subunit or, is identified as multiple functionally-active amino acids, and said mutagenesis is effected at said single amino acid or each said amino acid and said mutagenesis is effected at each said amino acid.
18. The method claimed in claim 16 or 17, characterized in that the substitution step is effected by conjugation or by high voltage electroporation.

## FIG. 1.

## AMINO ACID SEQUENCE OF RADIO LABELLED PEPTIDES

CYCLE NO.	5	10	15
PEPTIDE A	Ile Leu Ala Gly Ala Leu Ala Thr Tyr Glu Ser *		Tyr Leu Ala
PEPTIDE B	Ile Leu - Gly Ala Leu Ala Thr Tyr Glu Ser *		Tyr Leu Ala
SUBUNIT S1	Ile Leu Ala Gly Ala Leu Ala Thr Tyr Glu Ser Glu Tyr Leu Ala		
RESIDUE NO.	120	125	130

\* UNCHARACTERIZED RADIOACTIVE AMINO ACID FOUND AT CYCLE 12

- NO AMINO ACID WAS IDENTIFIED AT THIS CYCLE

FIG.2.

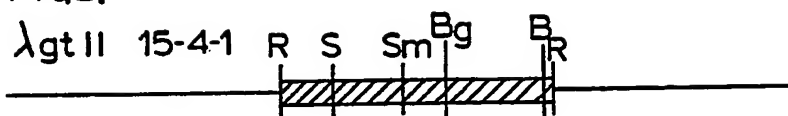


FIG.2B.

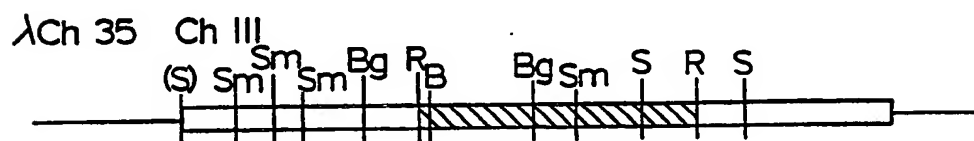


FIG.2C.

$\lambda$ Ch 35 Ch121/Ch411

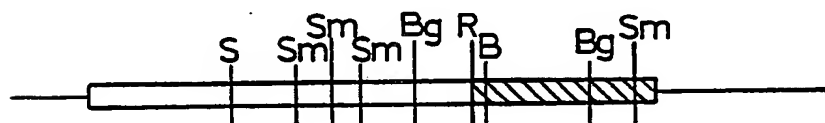


FIG.2D.

$\lambda$ Ch 35 Ch 431

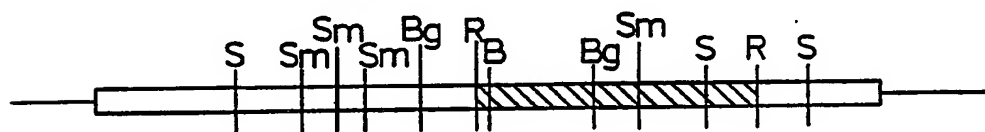


FIG.2E.

$\lambda$ Ch 35 Ch 421

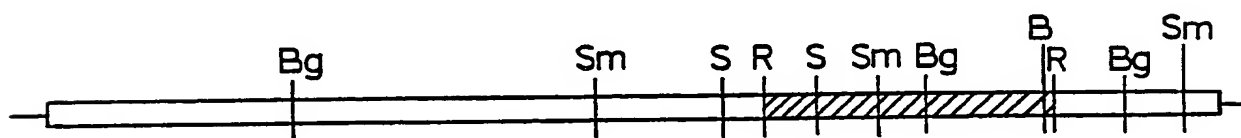
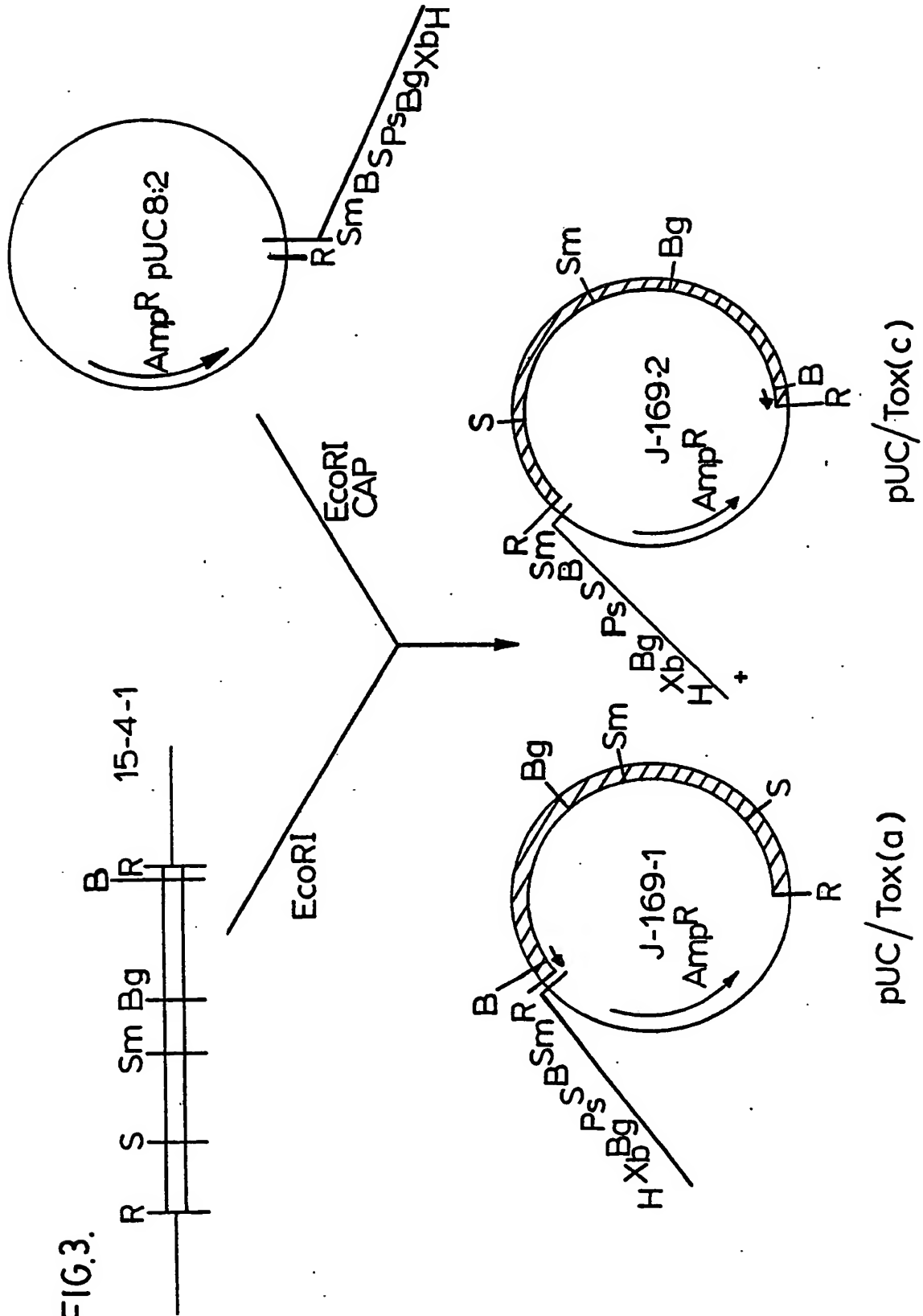




FIG.3.



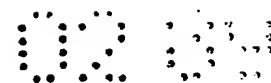


FIG.4A.

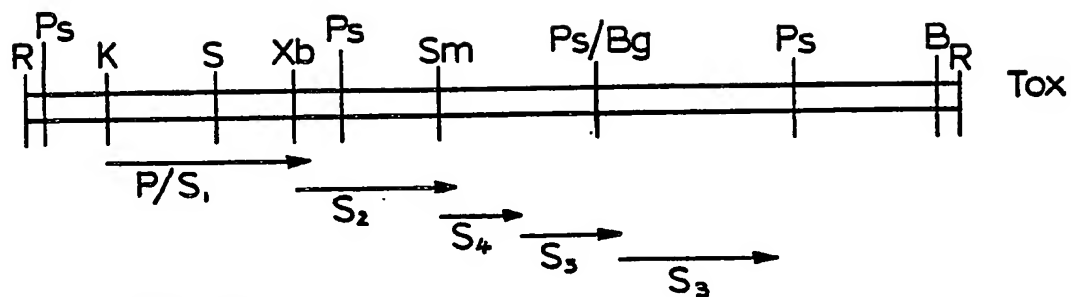


FIG.4B.

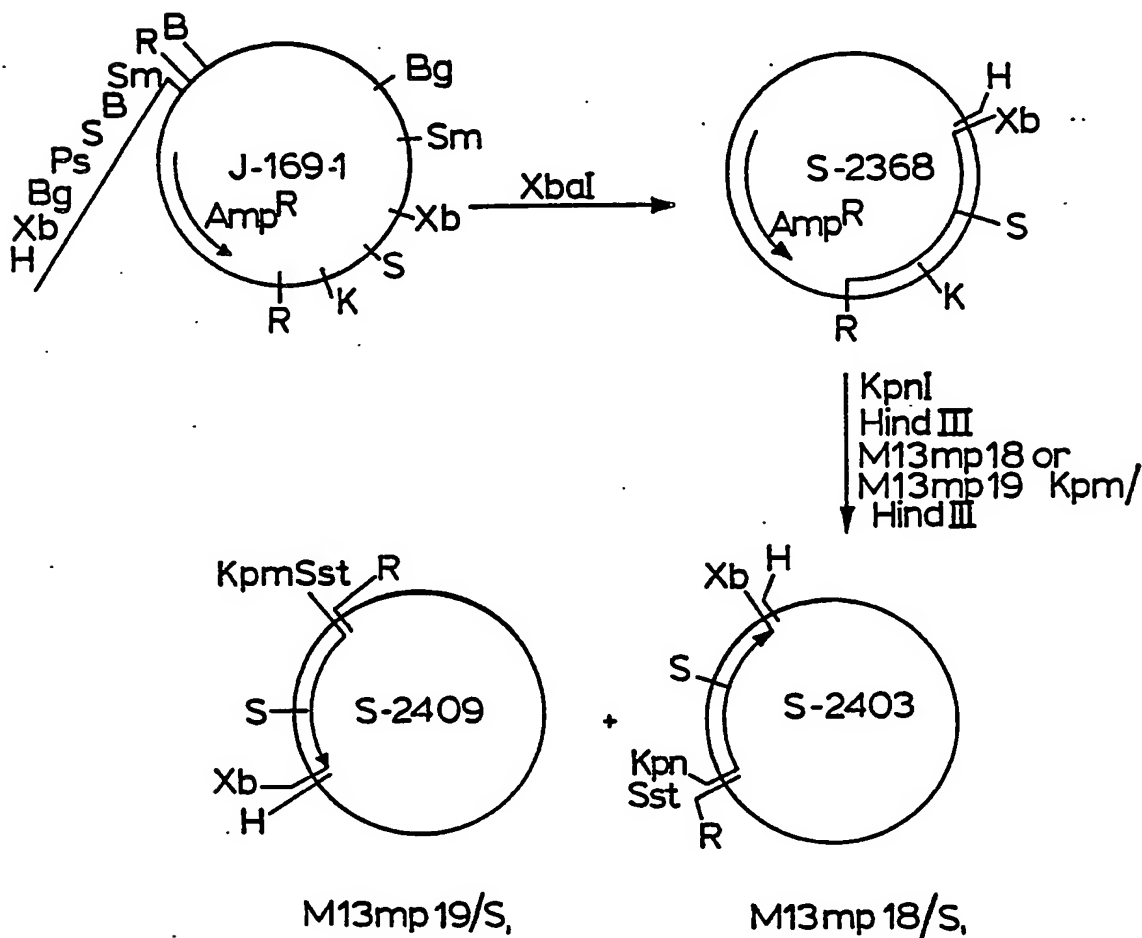




FIG.4C.

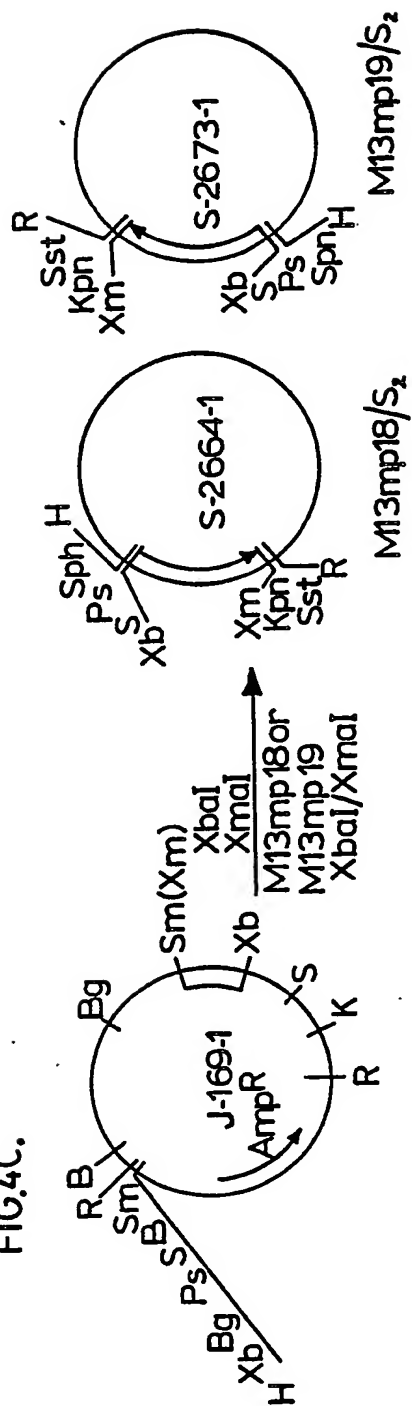
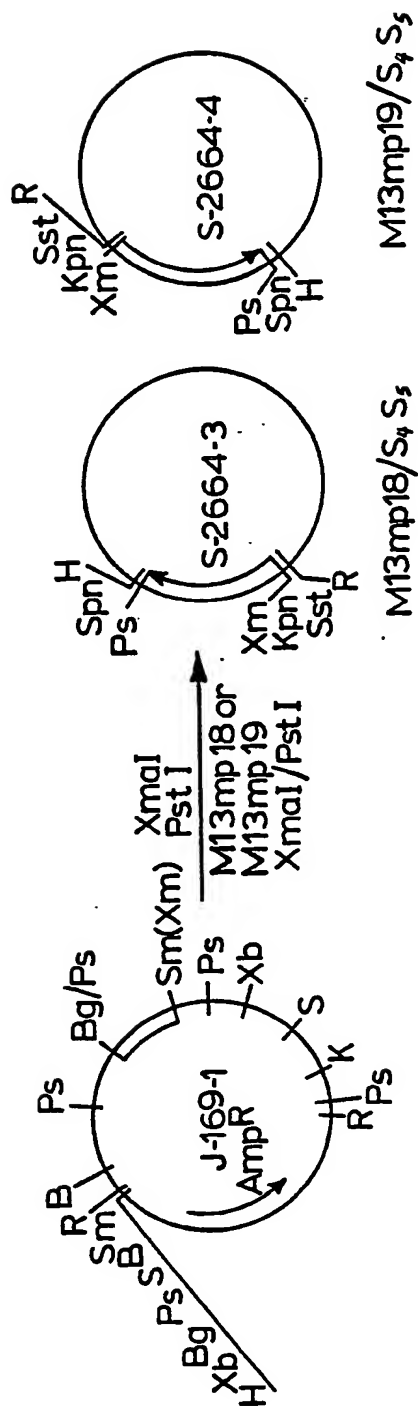
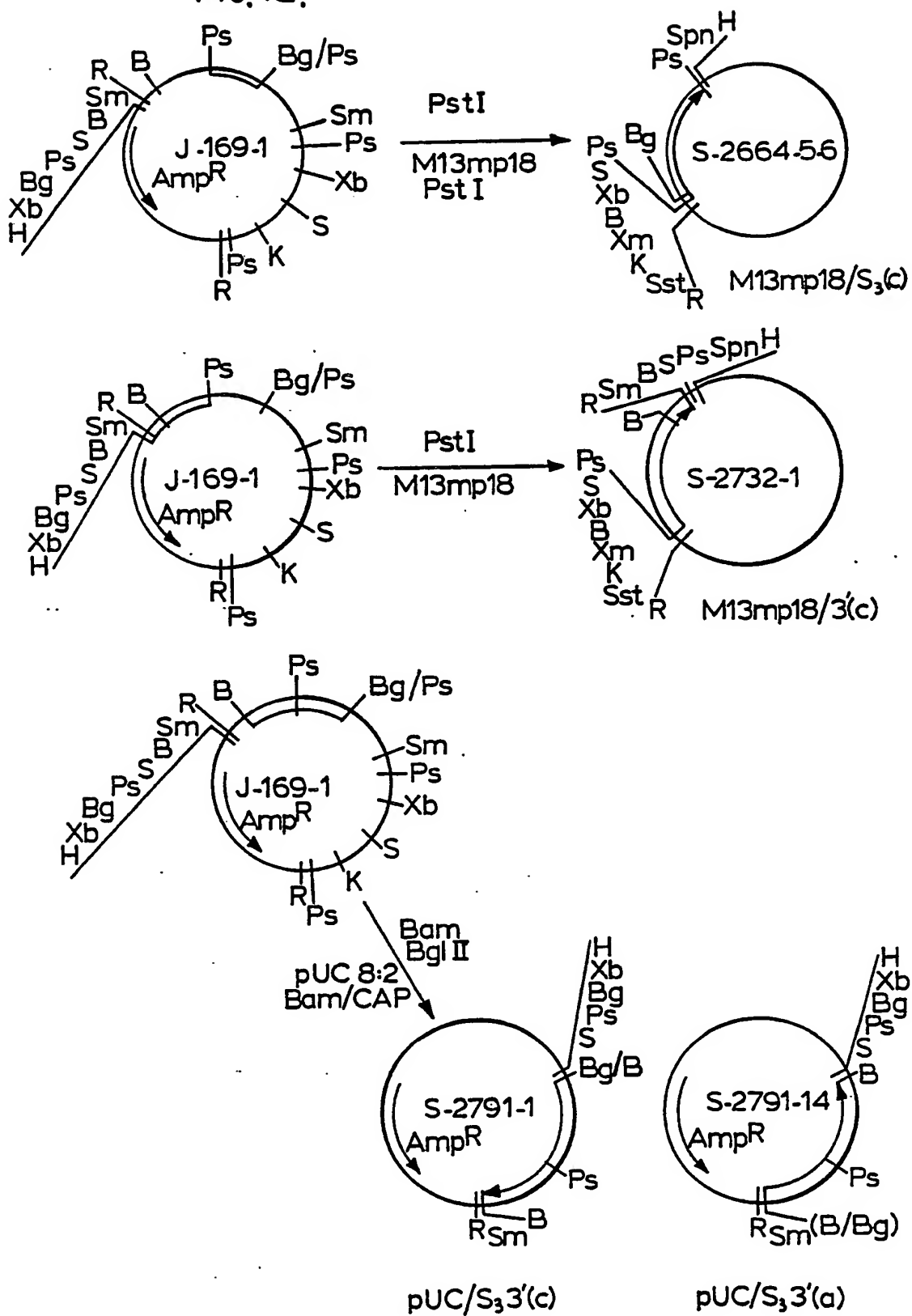


FIG.4D.



27 02 89

FIG.4E.



[illegible]

66C T66 CT6 AC6 T66 ATT CTT 66C 6TC ACS 6CB CCC 6TG ACT TCB CCB 6CA T66 66C 6AC 6AT CCT 66C 6CC ACC 6TA  
GLY TRP LEU THR TRP LEU ALA ILE LEU ALA VAL THR ALA PRO VAL THR SER PRO ALA TRP ALA ASP PRO PRO ALA THR VAL

TAC CSC TAT GAC TCC CGC CGC CCG GAG GAC GTT TTC CAG AAC GGA TTC ACG GCG TCG GGA AAC AAC GAC AAT GTG CTC GAA CAT  
Tyr Arg Tyr Asp Ser Arg Pro Pro Glu Asp Val Phe Gln Asn Gly Phe Tyr Ala Trp Gly Asn Asp Asn Val Leu Glu His  
8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35

[illegible]

CTC GAA CAT CCG ATG CAG GAA GCG GTC GAG GCG GAA CCG GCG GCG AGG GCG ACC GCG CAC TTC ATC GCG TAC ATC TAC GAA GTC  
LEU GLU HIS ARG MET GLN GLU ALA VAL ALA GLU ALA ARG ALA GLY ARG GLY THR GLY HIS PHE ILE GLY TYR ILE TYR GLU VAL  
64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91

[illegible]

GCC GGC GGG GTG GCC ACC TAC CAG AGC GAA TAT CTG GCA CAC CGG CGC ATT CCG CCC GAA AAC ATC CGC AGG GTA ACG CGG GTC  
AALA GLY ALA LEU ALA THR TYR GLN SER GLU TYR LEU ALA HIS ARG ARG ILE PRO PRO GLU ASN ILE ARG ARG VAL THR ARG VAL  
120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147

[illegible]

AAAC CCC TAC ACA TCG CGA AAG TCC GTA GCG TCG ATC GTC GGC ACA TTG GTG CCG ATG CCG CCG GTG GTG GGC GCT TGC ATG GCG  
ASH PRO TYR THR SER ARG ARG SER VAL ALA SER ILE VAL GLY THR LEU VAL ARG MET ALA PRO VAL VAL GLY ALA CYS MET ALA  
1178 177 178 180 181 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198 199 200 201 202 203

CGG CAG GCG GAA AGC TCC GAG GCC ATG GCA GCG YGG TCC GAA CCG GCC GAG GCG ATG GTT CTC GTG TAC TAC GAA AGC ATC

GCG TAT TCG TTC TAGACCTGGCCCAAGCCCGGCCCAACTCCG56TAATTGACACG ATG CCG ATC GAC CCG AAG ACG CTC TGC CAY CTC CTG TCC  
 ALA TYR SER PHE  
 232 233 234 235  
 PHEI PRO ILE ASP ARG LYS THR LEU CYS HIS LEU LEU SER  
 52

6TT C18 C58 T18 62C CTC C6A TCT CAC 6T8 6CG 6CC TCC ACB CCA 6GC ATC 6TC ATT C69 C68 6AA CAG ATT ACC  
YAL LEU PRO LEU ALA LEU LEU GLY SER HIS VAL ALA ARG ALA SER THR PRO GLY ILE VAL ILE PRO PRO GLN GLU GLN ILE THR

GLN HIS GLY SER PRO TYR GLY ARG CYS ALA ASN LYS THR ARG ALA LEU THR VAL ALA GLU LEU ARG GLY SER GLY ASP LEU GLN

FIG.5(ii).

GAG TAC CTG CGT CAT GTG ACG CGC TGG TCA ATA TTT GCG CTC TAC GAT GGC ACC TAT CTC GGC GGC GAA TAT GGC GGC GTG  
 GLU TYR LEU ARG HIS VAL THR ARG GLY TRP SER ILE PHE ALA LEU TYR ASP GLY THR TYR LEU GLY GLY TYR GLY VAL  
  
 ATC AAG GAC GGA ACA CCC GGC GCA TTC GAC CTG AAA ACG ACG TTC TGC ATC ATG ACC ACG CGC AAT ACG GGT CAA CCC GCA  
 ILE LYS ASP GLY THR PRO GLY GLY ALA PHE ASP LEU LYS THR THR PHE CYS ILE MET THR THR ARG ASN THR GLY GLN PRO ALA  
  
 ACG CAT CAC TAC TAC ACG AAC GTC ACC GGC ACT CGC CTG CTC TCC AGC ACC AAC ACG ACG CTA TGC GCG GTC TTC GTC AGA ACG  
 THR ASP HIS TYR TYR SER ASN VAL THR ALA THR ARG LEU LEU SER SER THR ASN SER ARG LEU CYS ALA VAL PHE VAL ARG SER  
  
 GGG CAA CCG GTC ATT GGC GGC TGC ACC ACG CGC TAT GAC GGC AAG TAC TGG AGC ATG TAC ACG CCG CTG CCG AAA ATG CTT TAC  
 GLY GLN PRO VAL ILE GLY ALA CYS THR SER PRO TYR ASP GLY LYS TYR TRP SER MET TYR SER ARG LEU ARG LYS MET LEU TYR  
  
 CTG ATC TAC GTG GGC GGC ATC TCC GTA CCG GTC CAT GTG ACG AAG GAA CAG TAT TAC GAC TAT GAG GAC GCA ACG TTC GAG  
 LEU ILE TYR VAL ALA GLY ILE SER VAL ARG VAL HIS VAL SER LYS GLU GLN TYR TYR ASP TYR GLU ASP ALA THR PHE GLU  
  
 ACT TAC GGC CTT ACC GGC ATC TCC ATC TGC ATC CCT GGA TCA TCC TTA TGC TGGACGCTTCCCGACTCGAACCCCGCCCGGACAGGCGCG  
 THR TYR ALA LEU THR GLY ILE SER ILE CYS ASN PRO GLY SER SER LEU CYS  
  
 CGCCCGCGGATCGCCG GTG CCG GGC CTG GCG TGG TTG CTG GCA TCC GGC GCG ATG ACG CAT CTT TCC CCG GGC CTG GGC GAT GTT CCT  
 PHE MET ARG ALA LEU ALA TRP LEU LEU ALA SER GLY ALA MET THR HIS LEU SER PRO ALA LEU ALA ASP VAL PRO  
 S4  
 TAT GTG CTG GTG AAG ACC AAT ATG GTG GTC ACC ACG GTA GGC ATG AAG CCG TAT GAA GTC ACC CCG ACG GCG ATG CTG GTC TGC  
 TYR VAL LEU VAL LYS THR ASN MET VAL VAL THR SER VAL ALA MET LYS PRO TYR GLU VAL THR PRO THR ARG MET LEU VAL CYS  
  
 GGC ATC GGC GGC AAA CTG GGC GGC GGC ACG ACG CCG GAG GCG CAC GTG CCG TTC TGC TGC GGC AAG GAT CTC AAG CGT CCC  
 GLY ILE ALA ALA LYS LEU GLY ALA ALA SER SER PRO ASP ALA HIS VAL PRO PHE CYS PHE GLY LYS ASP LEU LYS ARG PRO  
  
 GGC ACG AGT CCC ATG GAA GTG TTG CCG GGC GTC TTC ATG CAA CAA CCG CCG CTG CCG ATG TTT CTG GGT CCC AAG CAA CTC  
 GLY SER SER PRO MET GLU VAL MET LEU ARG ALA VAL PHE MET GLN GLN ARG PRO LEU ARG MET PHE LEU GLY PRO LYS GLN LEU  
  
 ACT TTC GAA GGC AAG CCC GCG CTC GAA CTG ATC CCG ATG GTC GAA TGC ACG GGC AAG CAG GAT TGC CCC TGA AGCGGAACCCC ATG  
 THR PHE GLU GLY LYS PRO ALA LEU GLU LEU ILE ARG MET VAL GLU CYS SER GLY LYS GLN ASP CYS PRO  
 S5  
 CAT ACC ATC GCA TCC ATC CTG TTG TCC GTG CTC GGC ATA TAC ACG CCG GCT GAC GTC GGC GGC TTG CCG ACC CAT CTG TAC AAG  
 HIS THR ILE ALA SER ILE LEU LEU SER VAL LEU GLY ILE TYR SER PRO ALA ASP VAL ALA GLY LEU PRO THR HIS LEU TYR LYS  
  
 AAC TTC ACT GTC CAG GAG CTG GGC TTG AAA CTG AAG GGC AAG AAT CAG GAG TTC TGC CTG ACC GGC TTC ATG TCG GGC AGA ACG  
 ASN PHE THR VAL GLN GLU LEU ALA LEU LYS LEU LYS GLY LYS ASN GLN GLU PHE THR ALA PHE MET SER GLY ARG SER  
  
 CTG GTC GCG GCG TGC CTG TCC GAC GCG GGA CAC GAG CAC ACG TGG TTC GAC ACC ATG CTT GGC TTT GGC ATA TCC GCG TAT  
 LEU AVL ARG ALA CYS LEU SER ASP ALA GLY HIS GLU HIS ASP THR TRP PHE ASP THR MET LEU GLY PHE ALA ILE SER ALA TYR  
  
 GCG CTC AAG ACG GCG ATC GCG CTG ACG GTG GAA CAC TCG CCG TAT CCG GGC ACT CCC GGC GAT CTG CTC GAG CTG CAG ATC TGC  
 ALA LEU LYS SER ARG ILE ALA LEU THR VAL GLU ASP SER PRO TYR PRO GLY THR PRO GLY ASP LEU LEU GLN ILE CYS

CCG CTC AAC GSA TAT TGC GAATGAACCTTCGSEAGGTTTCGACGTTTCGCGCAATCCGCTTGAGAGCATCTTCGCGCTGTTCCATTCGSGAACACCGGAAAC  
PRO LEU ASN GLY TYR CYS GLU

ATG	CTG	ATC	AAC	AAG	AAG	CTG	CTT	CAT	CAC	ATT	CTG	CCC	ATC	CTG	GTC	CTC	GGC	ATG	CGC	ACG	GCC	GCC	CAG	GCC			
MET	LEU	ILE	ASN	ASN	LYS	LYS	LEU	LEU	HIS	HIS	ILE	LEU	PRO	ILE	LEU	VAL	LEU	ALA	LEU	GLY	MET	ARG	THR	ALA	GLN	ALA	
S3																											
GTT	GGC	CCA	GGC	ATC	ATC	ATC	CCG	CCG	AAG	GCA	CTG	TTC	ACC	CAA	CAG	GGC	GGC	GGC	TAT	GGA	CGC	TGC	CCG	AAC	GGA	ACC	CGC
VAL	ALA	PRO	GLY	ILE	VAL	ILE	PRO	PRO	LYS	ALA	LEU	PHE	THR	GLN	GLN	GLY	GLY	ALA	TYR	GLY	ARG	CYS	PRO	ASN	GLY	THR	ARG
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
S3																											
GCC	TTG	ACC	GTC	GAA	CTG	CGC	GGC	AAC	GCC	GAA	TTG	CAG	ACG	TAT	TTG	CGC	CAG	ATA	ACG	CCC	GGC	TGG	TCC	ATA	TAC	GGT	
ALA	LEU	THR	VAL	ALA	GLU	LEU	ARG	GLY	ASN	ALA	GLU	LEU	GLN	THR	TYR	LEU	ARG	GLN	ILE	THR	PRO	GLY	TRP	SER	ILE	TYR	GLY
29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
S3																											
CTC	TAT	GAC	GST	ACG	TAC	CTG	GGC	CAG	GGC	TAC	GGC	GGC	ATC	ATC	AAG	GAC	CGC	CCA	GGC	CGC	GGG	TTT	ATT	TAT	TAT	CGC	GAA
LEU	TYR	ASP	GLY	THR	TYR	LEU	GLY	GLN	ALA	TYR	GLY	GLY	ILE	ILE	LYS	ASP	ALA	PRO	PRO	GLY	ALA	GLY	PHE	ILE	TYR	ARG	GLU
57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84
S3																											
ACT	TTT	TGC	ATC	ACG	ATA	TAC	AAG	ACC	GGG	CAA	CCG	GCT	GGC	GAT	CAC	TAC	TAC	TAC	AGC	AAG	GTC	ACG	GCC	ACG	CGC	CTG	CTC
THR	PHE	CYS	ILE	THR	THR	ILE	THR	LYS	THR	GLY	GLN	PRO	ALA	ALA	ASP	HIS	TYR	TYR	SER	LYS	VAL	THR	ALA	THR	ARG	LEU	LEU
85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112
S3																											
GCC	AGC	ACC	AAC	AGC	AGG	CTG	TGC	GGC	GTA	TTT	GTC	AGG	GAC	GGG	CAA	TGG	GTC	ATC	GGA	GCC	TGC	GCC	AGC	CCG	TAT	GAA	GGC
ALA	SER	THR	ASN	SER	ARG	LEU	CYS	ALA	VAL	PHE	VAL	ARG	ASP	GLY	GLN	SER	VAL	ILE	GLY	ALA	CYS	ALA	SER	PRO	TYR	GLU	GLY
113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140
S3																											
AGG	TAC	AEA	GAC	ATG	TAC	GAC	CGC	CTG	CGG	CGC	CTG	CTG	TAC	ATG	ATC	TAT	ATG	TCC	GGC	CTT	GGC	GTA	CGC	GTC	CAC	GTC	AGC
ARG	TYR	ARG	ASP	MET	TYR	ASP	ALA	LEU	ARG	ARG	LEU	LEU	TYR	MET	ILE	TYR	MET	SER	GLY	LEU	ALA	VAL	ARG	VAL	HIS	VAL	SER
141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168
S3																											
AAG	GAA	GAG	CAG	TAT	TAC	GAC	TAC	GAG	GAC	GCC	ACA	TTC	CAG	ACC	TAT	GCC	CTC	ACC	GGC	ATT	TCC	CTC	TGC	AAC	CGC</		

FIG. 6A.

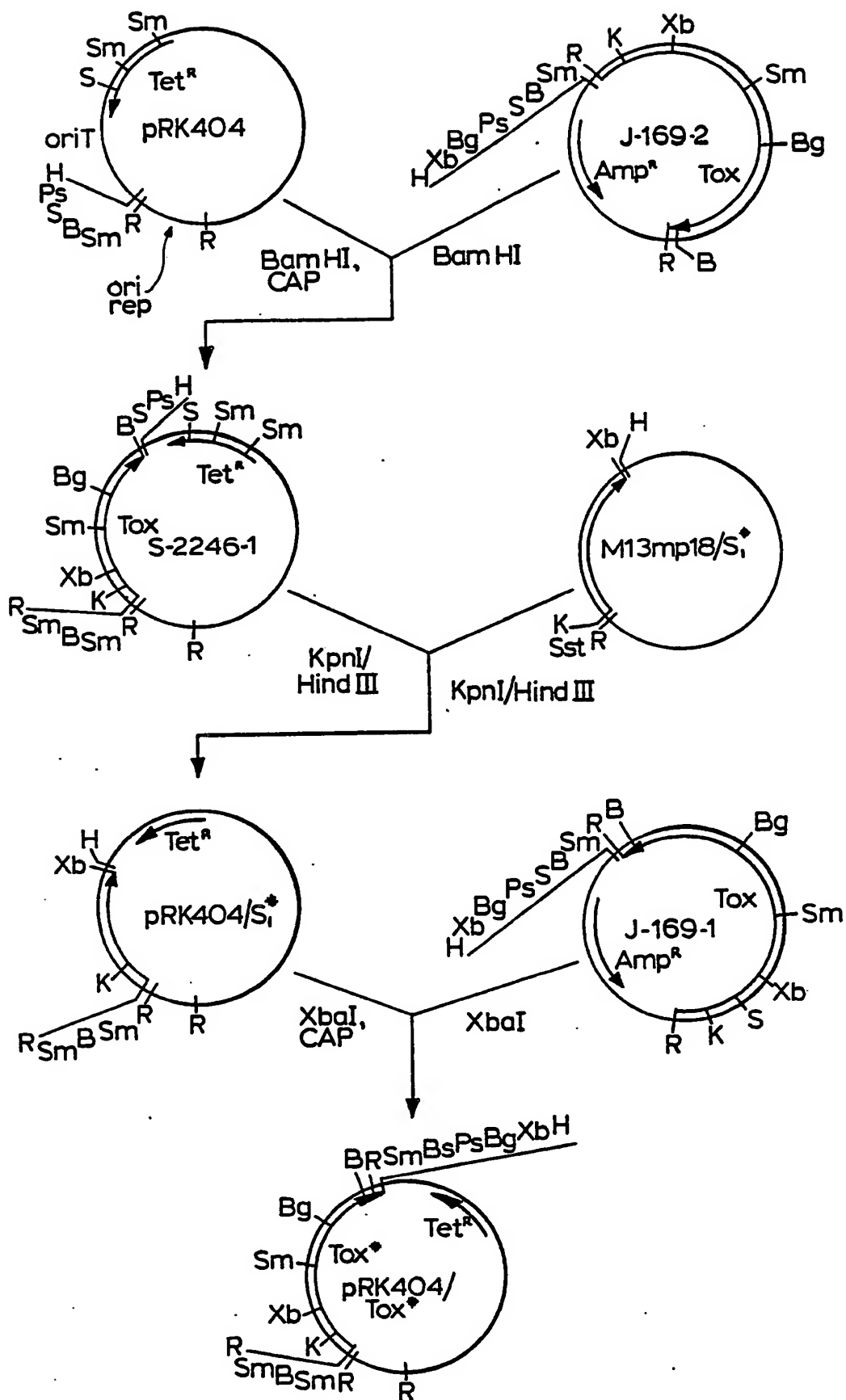




FIG. 6B.

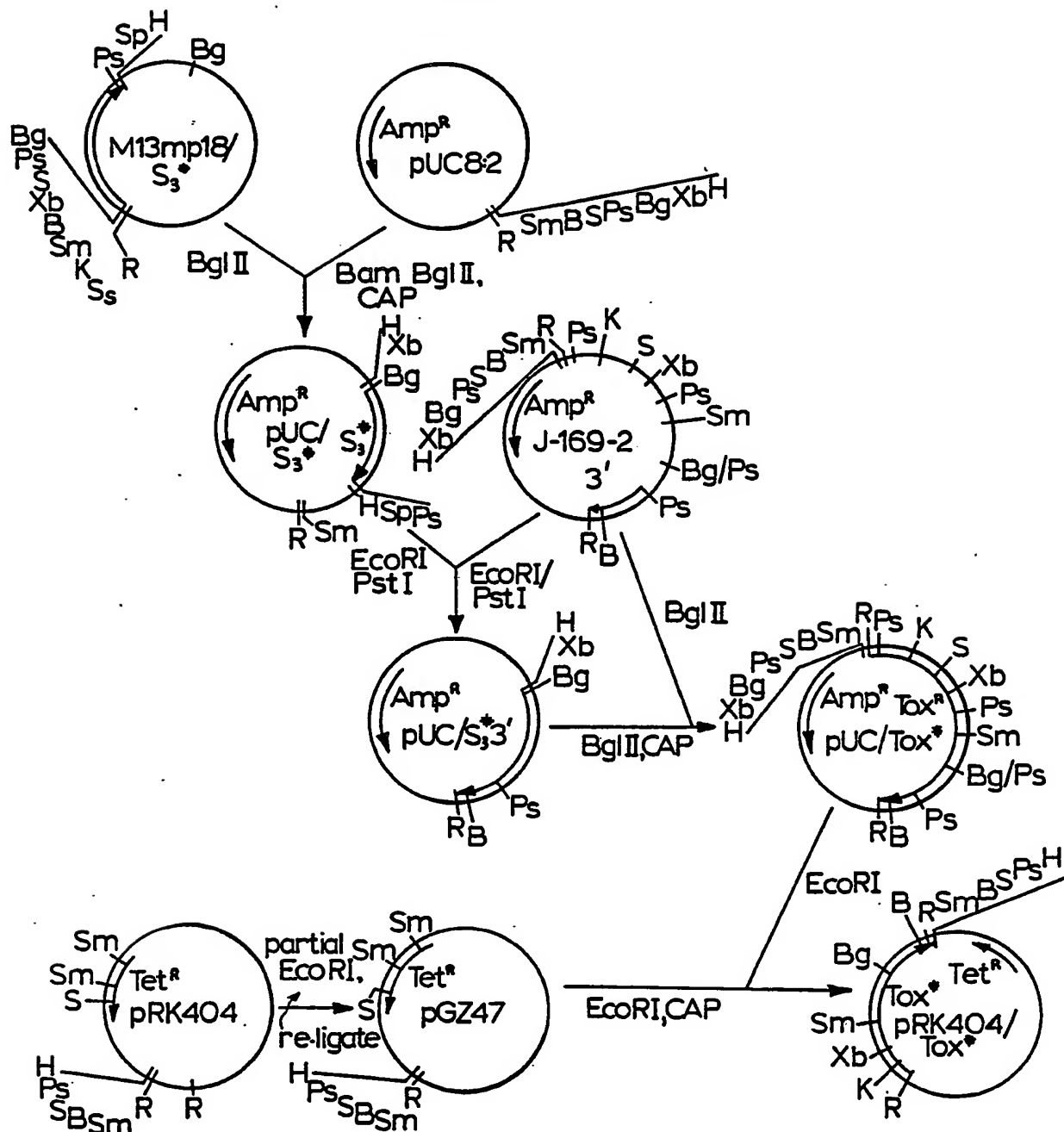
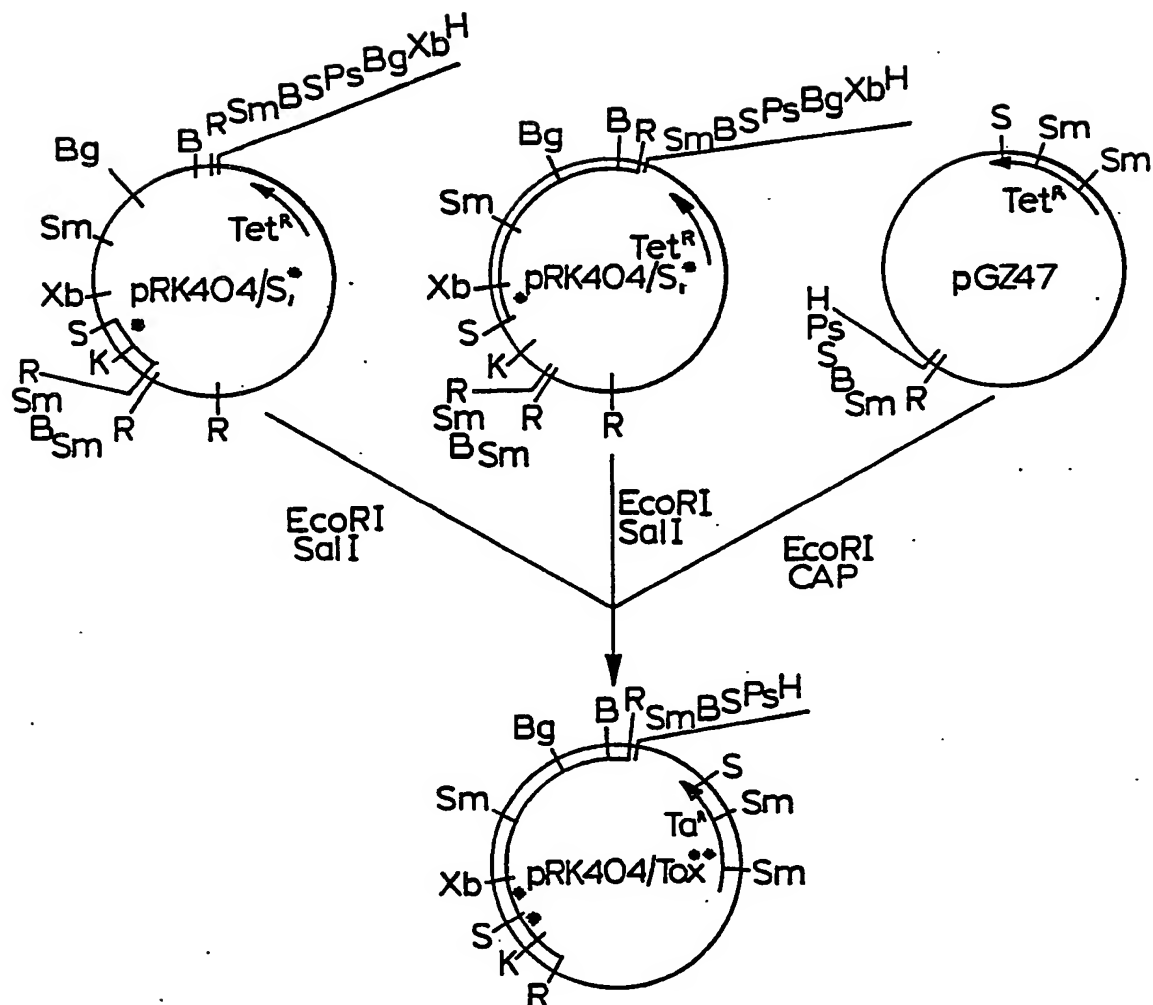


FIG.6C.



\*DENOTES THE LOCATION OF MUTATIONS IN THE S<sub>i</sub> GENE

FIG.7.



FIG.8A.

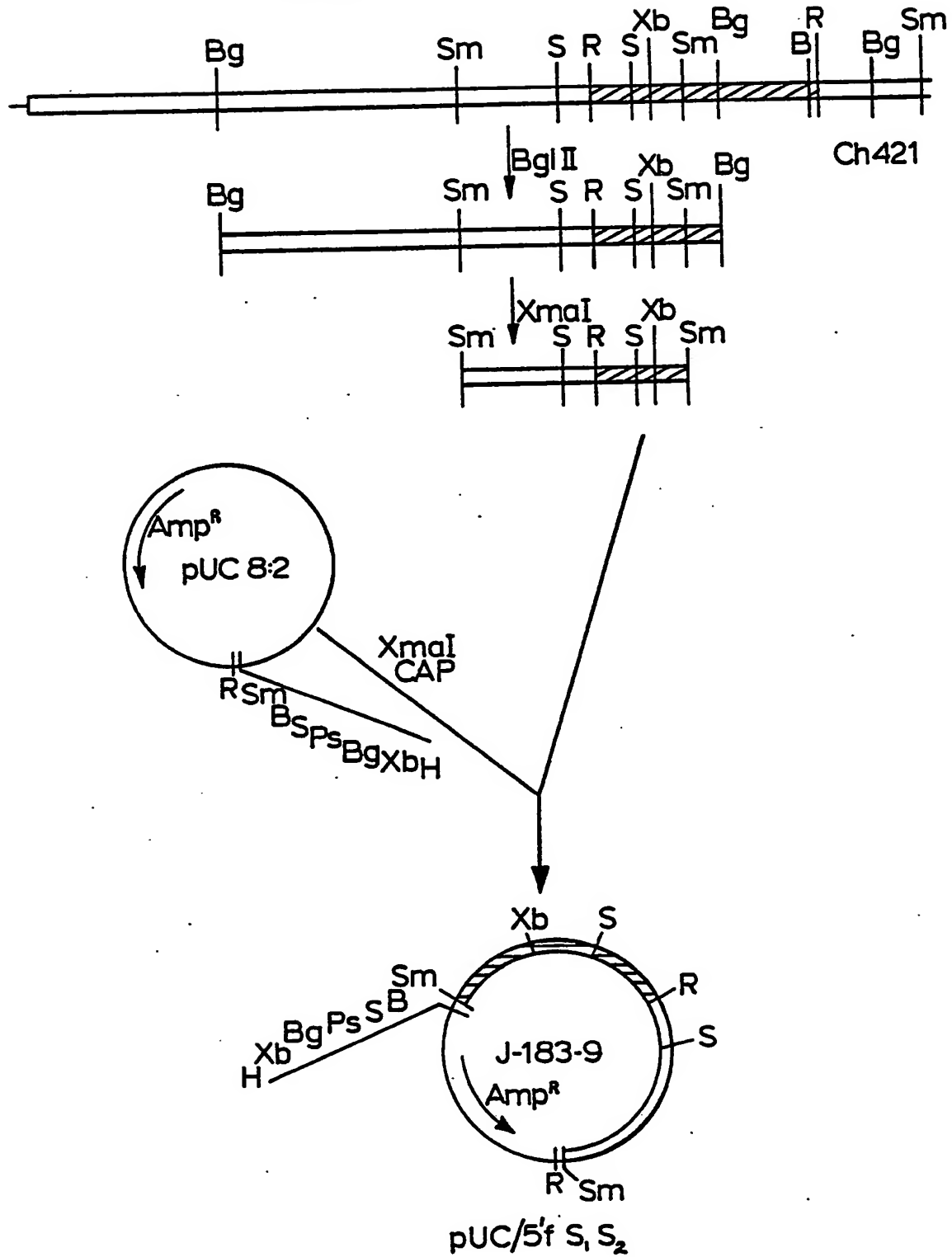


FIG.8B.

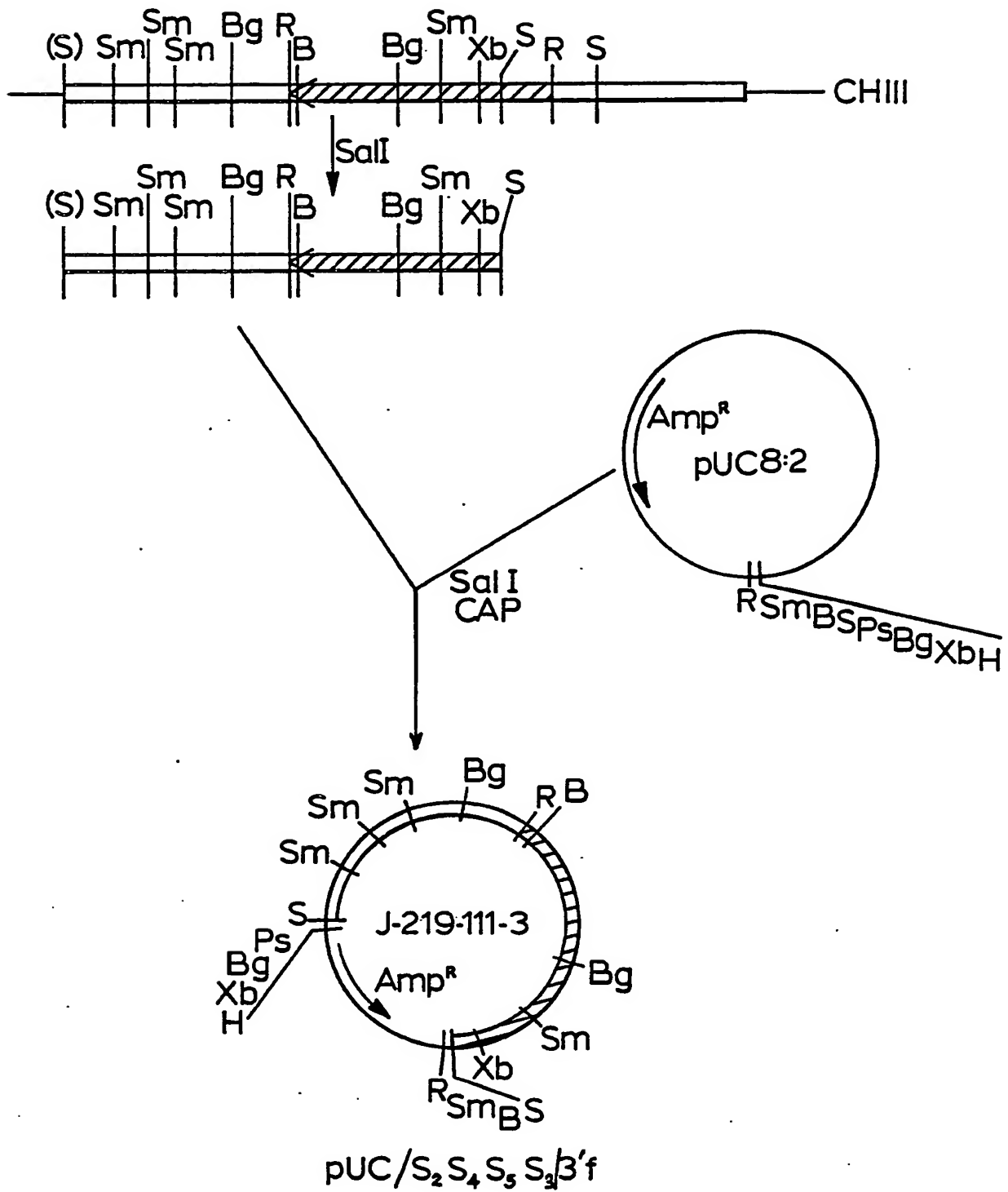


FIG.8C.

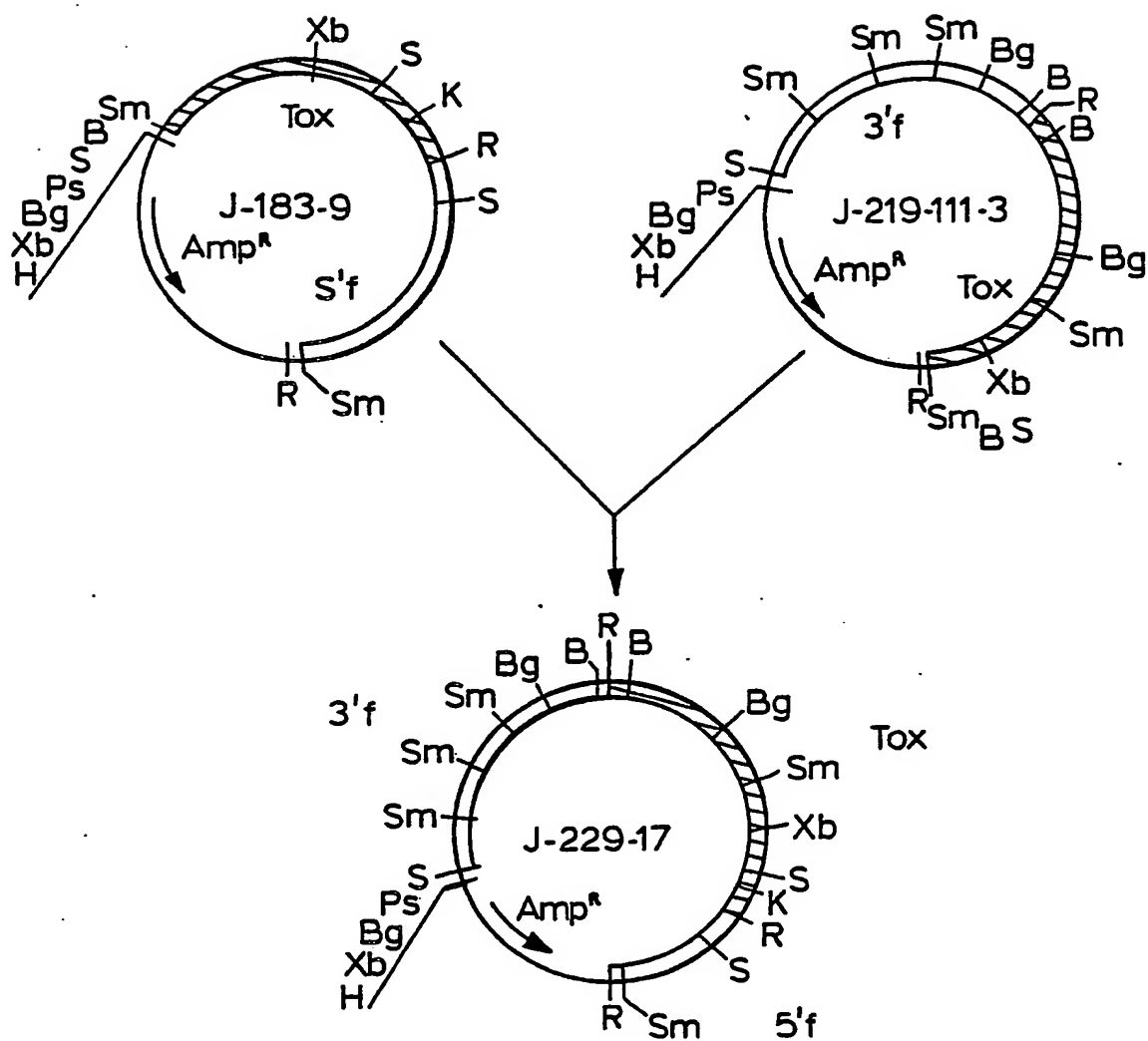


FIG. 9A.

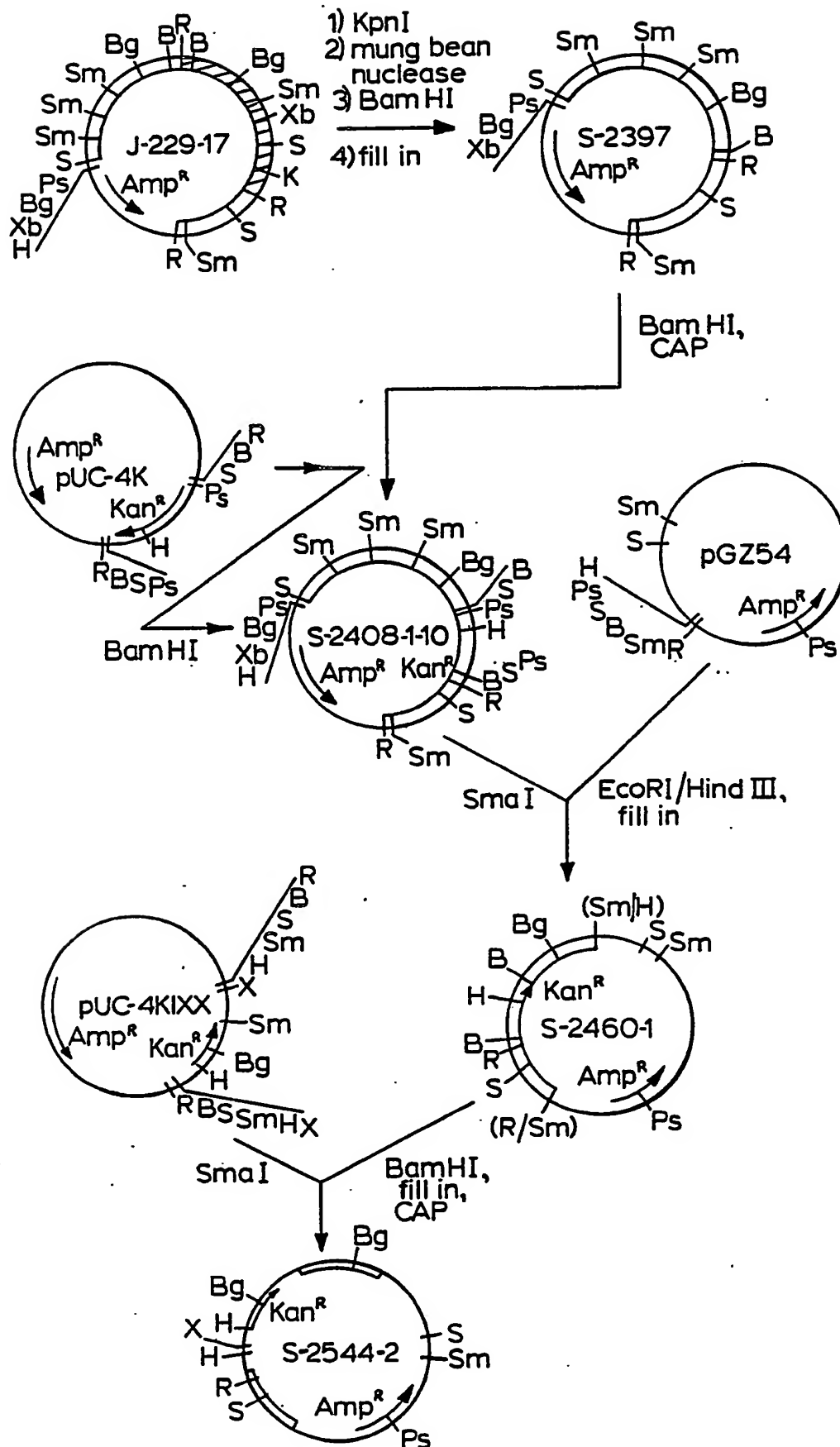


FIG. 9B.

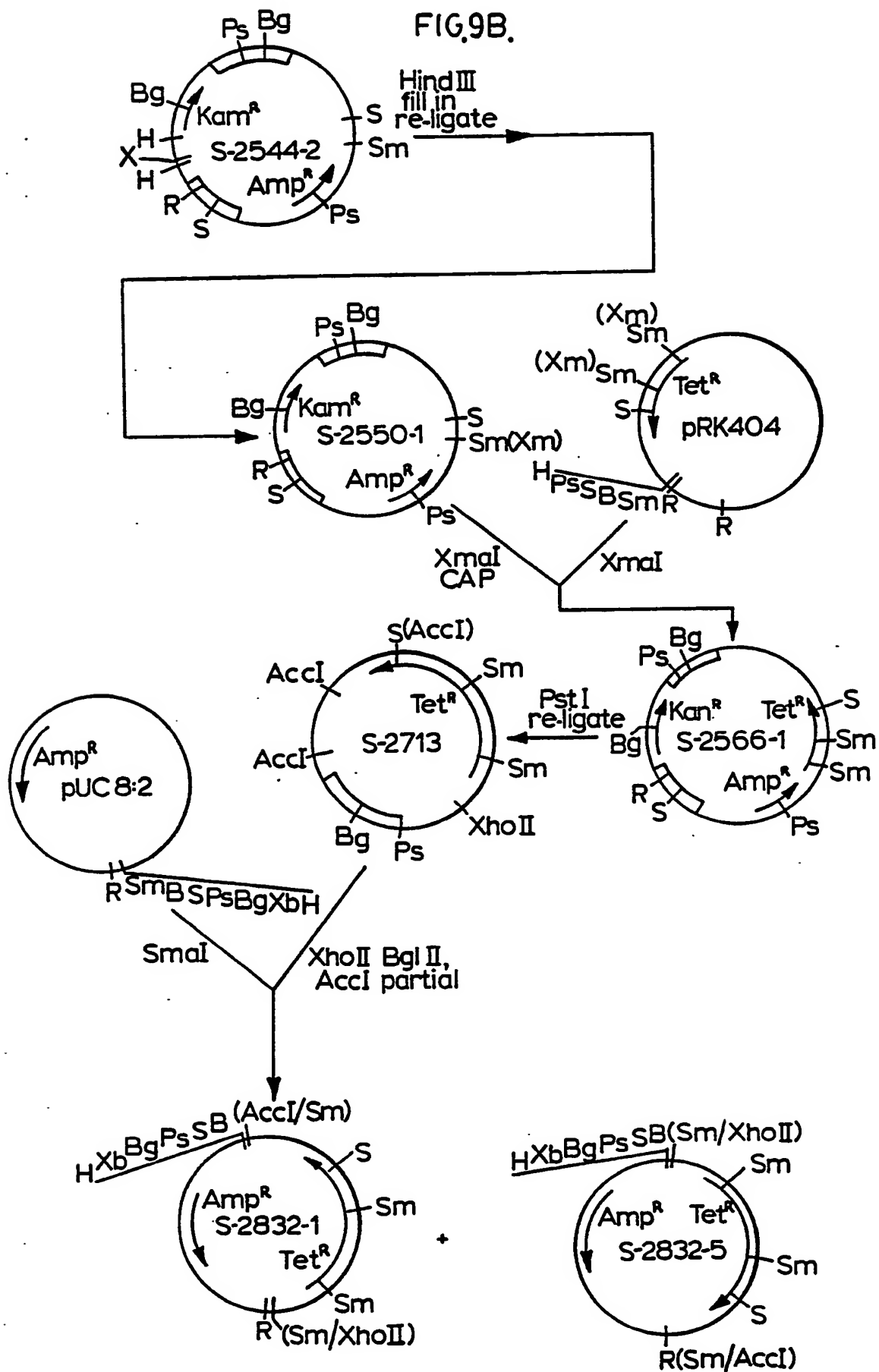




FIG. 9C.

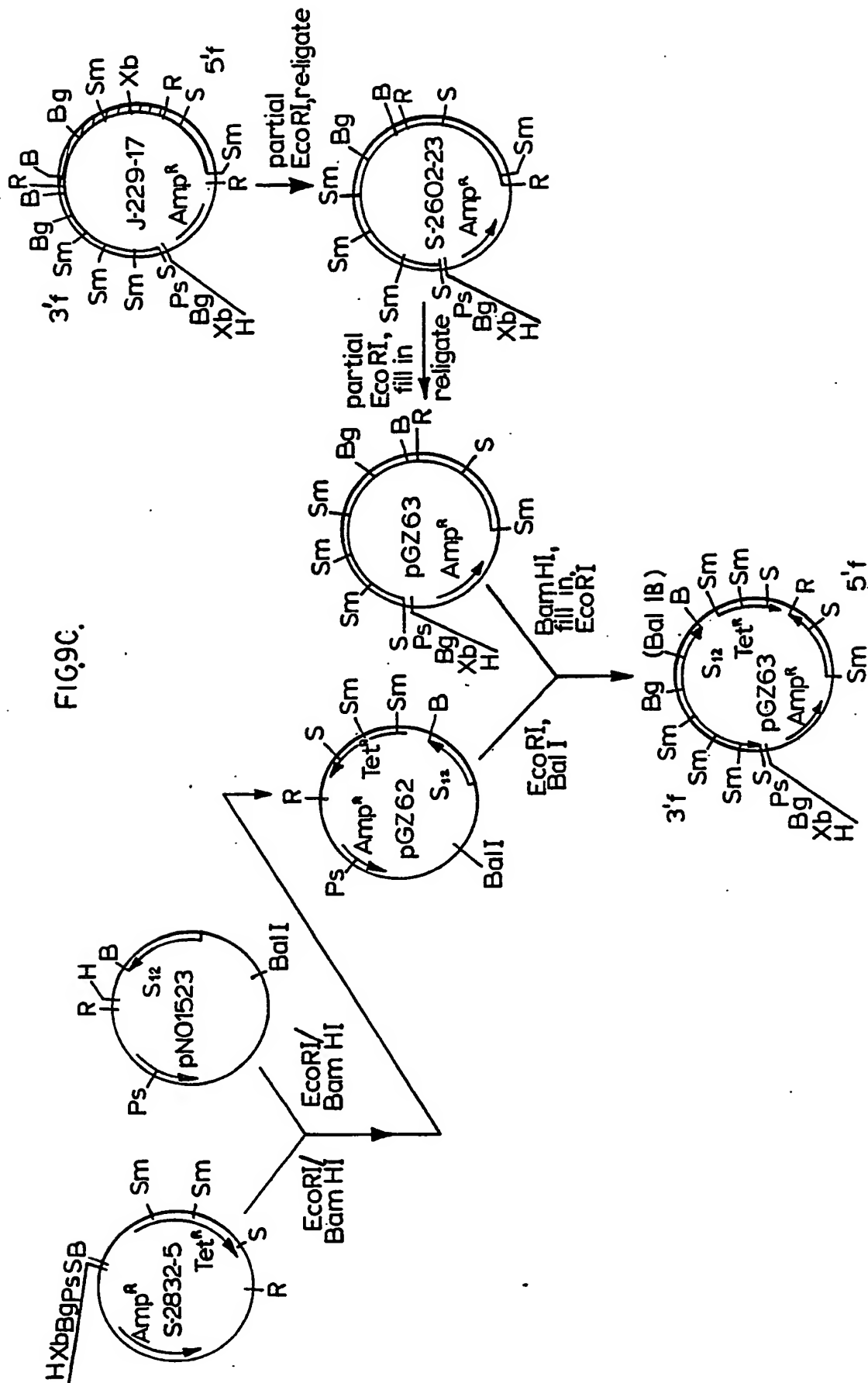


FIG.10.

